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PRINCIPAL INVESTIGATOR: Paloma H. Giangrande

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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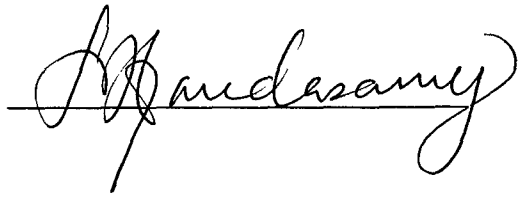

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13. ABSTRACT *(Maximum 200 words)*

In humans, the progesterone receptor exists as two isoforms, hPR-A and hPR-B. hPR-B is transcriptionally active, while hPR-A is inactive and acts as a transdominant repressor of estrogen receptor (ER) transcriptional activity. Although, the precise mechanism of hPR-A transrepression is not fully understood, we identified a domain located within the amino terminus of hPR-A, necessary for transrepression. This domain is contained within both PR isoforms, however, its activity is manifested only in hPR-A, suggesting that hPR-A interacts with a set of cofactors that are distinct from those recognized by hPR-B. In support of this hypothesis, we found that the interaction of hPR-A with the corepressor SMRT is stronger than that observed with hPR-B. The importance of such interaction, is demonstrated by using a dominant negative variant of SMRT to partially reverse hPR-A transrepression of ER activity, implicating SMRT in the transrepression by hPR-A. In addition, we show that hPR-B, but not hPR-A, interacts efficiently with the coactivators SRC-1 and GRIP1. Thus, the inability of hPR-A, in contrast to hPR-B, to recruit coactivators, as well as its strong association with corepressor proteins, correlates with the differences in the transcriptional activities of the two PR isoforms.

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Paloma Gonzalez 7/30/99

PI - Signature Date

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INTRODUCTION

The role of the estrogen receptor (ER) in breast cancer has been suggested both by its ability to stimulate cell proliferation as well as the observation that ER is expressed in 60% of primary breast tumor biopsies but only in 6% of normal breast tissue (1). Drugs which interfere with ER activity such as the antiestrogen Tamoxifen have been only partially successful in the treatment of breast cancers emphasizing the need for new targets as well as new pharmacological agents against these targets (2, 3, 4). The observation that antiprogestins such as RU486 could function as antiestrogens suggested that the progesterone receptor (PR) could be a potential target in the treatment of breast cancers (5, 6). To this effect our previous data show that the smaller isoform of human PR (hPR-A) functions as a ligand-dependent transdominant repressor of estrogen receptor (ER) transcriptional activity (7, 8). Although, the precise mechanism of hPR-A transrepression is not fully understood, we have recently identified an inhibitory domain (ID) located within the amino terminus of hPR-A, which permits hPR-A to transrepress ER transcriptional activity (9). Interestingly, although ID is contained within both PR isoforms, its activity is manifested only in the context of hPR-A, suggesting that hPR-A interacts with a set of cofactors that are distinct from those recognized by the larger isoform, hPR-B. To investigate potential role(s) of differential cofactor interactions, we looked at the ability of hPR-A and hPR-B to associate with different coactivators and corepressors and assessed the effect of these associations on the receptors' transcriptional activity (10). We also investigated whether any of these factors could be implicated in hPR-A-mediated transrepression of hER transcriptional activity (10). The goal of this project was to elucidate the mechanism of hPR-A transdominant repression by characterizing potential hPR-A-interacting partners which are necessary for ER transcriptional activation. We anticipate that new pharmacological agents against these targets could be used to treat breast cancers which currently escape endocrine intervention.

BODY

I. Identification of an inhibitory domain within hPR-A required for transdominant repression of ER transcriptional activity.

Human PR exists as two functionally distinct isoforms hPR-A and hPR-B (11). hPR-A is a truncated form of hPR-B lacking amino acids 1-164. In most cell- and promoter-contexts, hPR-B functions as a transcriptional activator, while hPR-A is transcriptionally inactive and functions as a ligand-dependent transdominant repressor of ER transcriptional activity (7, 8, 9). Unlike hPR-A, the A isoform of the chicken progesterone receptor (cPR-A), which shares 70% sequence homology with hPR-A, lacks this transdominant repressor function and acts as a strong activator of transcription (9). We have observed that the most extensive differences between the primary structures of the chicken and human PR-As are found in the amino terminal domains. Deletion of the first 140 amino acids from hPR-A (ΔhPR-A) (Figure 1a) converted hPR-A into a transcriptional activator (Figure 1b) and abolished its ability to transrepress ER transcriptional activity (Figure 1c) suggesting that this domain is necessary for hPR-A transdominant repression (9). In addition, we found that this domain does not have autonomous activity when fused to a heterologous DBD suggesting that other sequences present within PR may be required for transrepression (9) (Figure 2).

II. The amino termini of hPR-A and hPR-B interact differentially with the carboxyl terminus of PR (hLBD) implying different receptor conformations.

The presence of an inhibitory domain within human PR, whose function is masked in hPR-B, but not in hPR-A, suggests that the two receptor isoforms display different conformations within the cell which may allow for different cofactor interactions. This hypothesis is supported by our recent studies which analyzed the ability of separately expressed N- (PR-A and PR-B) and C-domains [hinge region plus ligand binding domain (hLBD)] of PR to interact in cells, by a mammalian two hybrid assay, and *in vitro* using purified expressed domains of PR (12). Specifically we found that the amino terminus of hPR-B, but not that of hPR-A, interacts efficiently with its hLBD both *in vivo* (Figure 3) and *in vitro* (Figure 4) in an agonist-dependent manner and does not interact in the presence of antagonist RU486 (12). Together, these results suggest that the interaction between N- and C-terminal domains of PR is direct and requires an agonist induced conformational change in the LBD that is not allowed by antagonists. In addition, the more efficient interaction of the N-terminus of hPR-B, but not that of hPR-A, with the hLBD suggests that distinct structural differences between N- and C-terminal regions of hPR-A and hPR-B contribute to functional differences between hPR-A and hPR-B.

III. The two progesterone receptors exhibit different cofactor interactions which may explain the differences in their transcriptional activities.

To determine whether the structural differences between the two receptors allow the receptors to interact with different cofactors, we looked at the ability of hPR-A and hPR-B to interact with various coactivators and corepressors (10). We demonstrated using a combination of *in vitro* and *in vivo* methodologies that the two receptors exhibit different cofactor interactions. Specifically, we showed using the mammalian two hybrid assay that the carboxyl terminus of the corepressor SMRT (C'SMRT), but not that of the corepressor NCoR (ΔN4), interacts more strongly with hPR-A, than with hPR-B, and that this interaction is facilitated by ID (Figure 5). The physiological significance of this interaction was demonstrated using the dominant negative variant of SMRT, C'SMRT, to partially reverse hPR-A transdominant repression of ER transcriptional activity, directly implicating SMRT in the transrepresion of ER activity by hPR-A. This was done by cotransfecting HeLa cells with ER, PR-A, and increasing concentrations of C'SMRT, ΔN4, or full length SMRT in the presence of estradiol and RU486 (Figure 6). Increasing concentrations of full length SMRT did not reverse transrepression of ER activity by hPR-A (data not shown). In addition, we show that hPR-A, unlike hPR-B, is unable to efficiently recruit the transcriptional coactivators GRIP-1 and SRC-1 in the presence of agonist but not antagonists (10). This was determined by using the mammalian two hybrid assay and assessing the ability of the nuclear receptor interacting domains (NR) of SRC-1 and GRIP-1 fused to Gal4 DNA binding domain to interact with PR-A or PR-B fused to VP16 (Figure 7). We concluded from the above data that the inability of hPR-A, in contrast to hPR-B, to recruit coactivators, as well as its strong association with corepressor proteins, correlates with the differences in the transcriptional activities of the two PR isoforms.

IV. Ongoing Studies

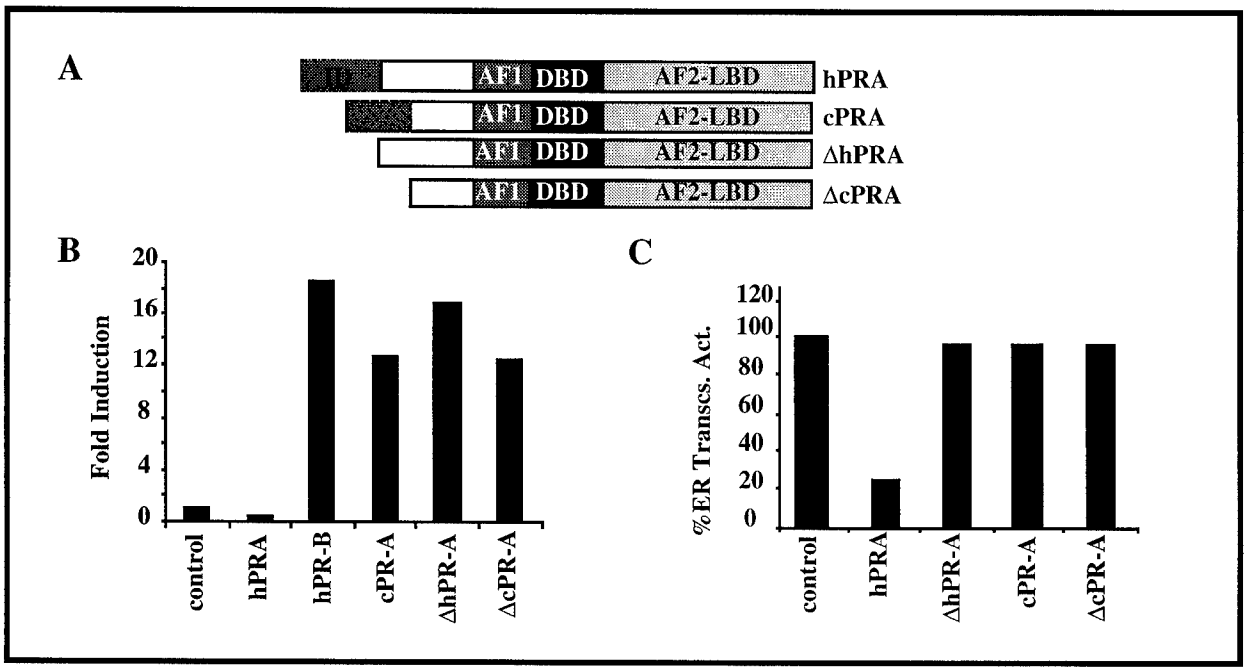
Previously, we had proposed to use a modified version of the yeast two-hybrid screen to identify possible interacting partners of hPR-A, responsible for hPR-A transdominant repression of ER transcriptional activity (13). In order to do this, we integrated two PRE elements upstream of a LacZ gene into the yeast genome by homologous recombination and used full-length hPR-A as a bait, given the importance of receptor context for hPR-A mediated transrepression of ER activity (9). Unfortunately, when we tested for the intrinsic transcriptional activity of our bait construct we found that it to be high in most yeast strains tested, both in the presence of agonist R5020 and antagonist RU486 (data not shown). In addition to exhibiting high basal activity, our bait was also toxic to the yeast, when expressed at high levels. For these reasons, we decided to abandon the yeast two-hybrid screen and make use of phage display technology to look for potential peptides that distinguish between the two receptors.

Phage display technology has been used successfully in the past to search for peptide sequences that mimic endogenous protein-protein interactions (14, 15). We have used this technology successfully in the laboratory to screen for ER-interacting motifs using random peptide libraries (16). In order to identify peptides which bound specifically to hPR-A we screened six different random peptide libraries (L14, L15, L16, L17, L18, L19) against full-length hPR-A bound to R5020, purified from baculovirus. We immobilized 4nmoles of hPR-A onto 96-well plates. BSA was used as a negative control. Following this step, phage expressed peptides, from a random peptide library, were added to the wells and allowed to incubate for 1h at 25°C. The wells were then washed to remove any unbound phage. The bound phage from each library were eluted using a low pH buffer and saved for plaque purification. After plaque purifying the phage from each individual library we isolated the PR-A-specific phage using a phage ELISA assay to screen against both hPR-A and hPR-B in the presence of R5020. An anti-M13 antibody coupled to HRP was used to determine the specificity of the interaction. From this screen we have isolated phage which 1) bind specifically to hPR-A, and 2) bind to both hPR-A and hPR-B (Figure 8). We are currently sequencing the individual phage to obtain PR-A-interacting sequences which will be used to search the protein database.

V. Conclusions

The data presented within clearly explain why hPR-B acts a transcriptional activator of progesterone responsive promoters and why hPR-A is transcriptionally inactive. However, it remains to be determined how hPR-A and SMRT work to repress ER transcriptional activity. In conclusion, we believe that the structural differences between hPR-A and hPR-B may allow the A isoform of the receptor to interact with factors, which are not recognized by hPR-B, to form a complex which can interfere with ER-mediated transcription. Formal proof of this hypothesis awaits the identification of factors which can distinguish between the two isoforms of the human progesterone receptor.

FIGURES



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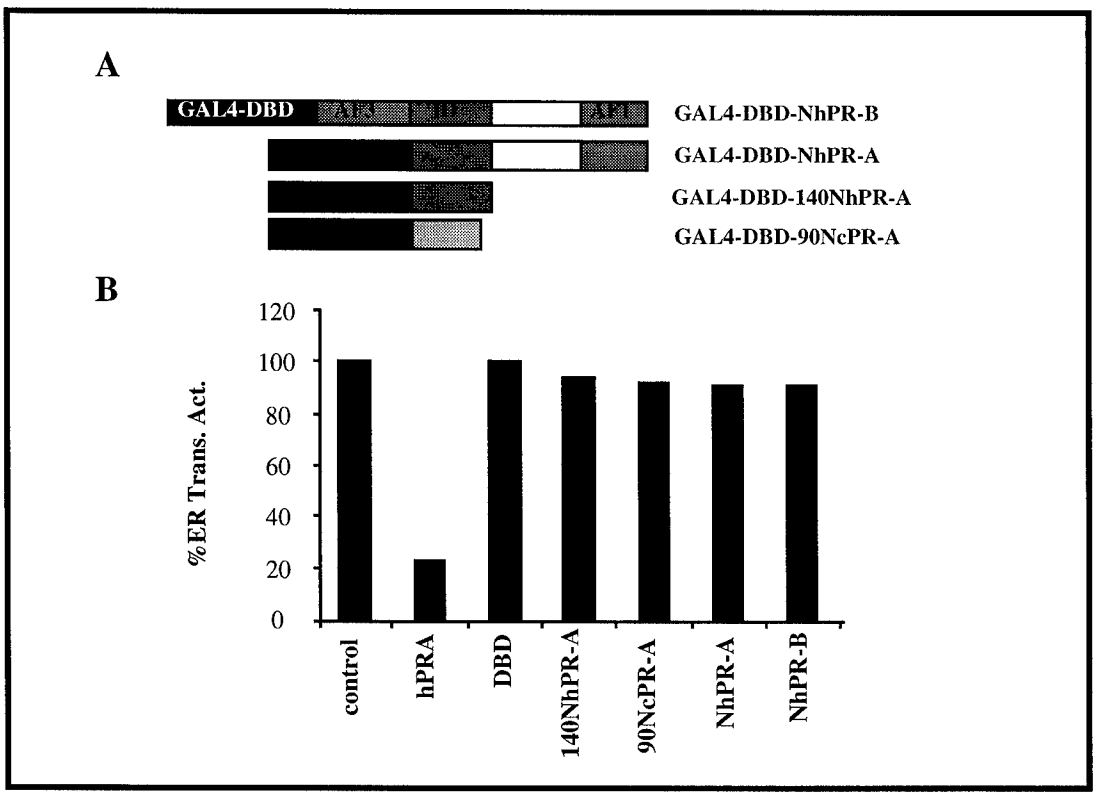

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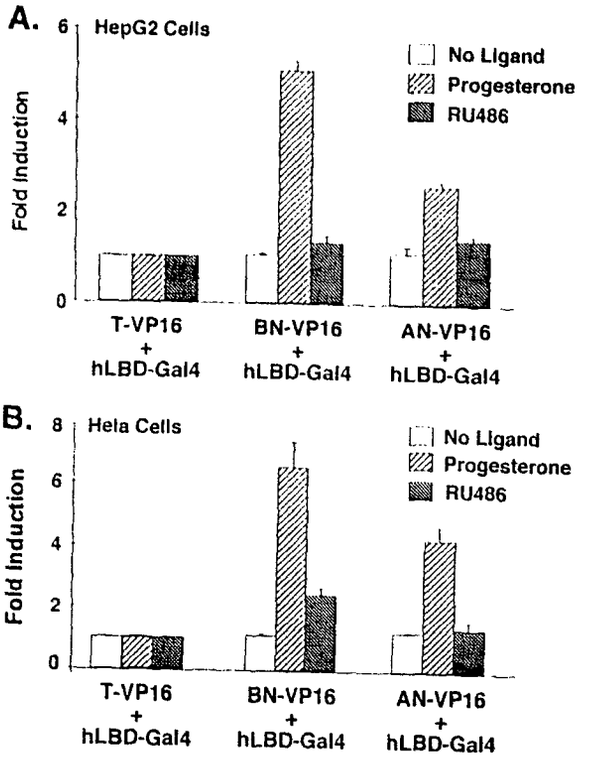

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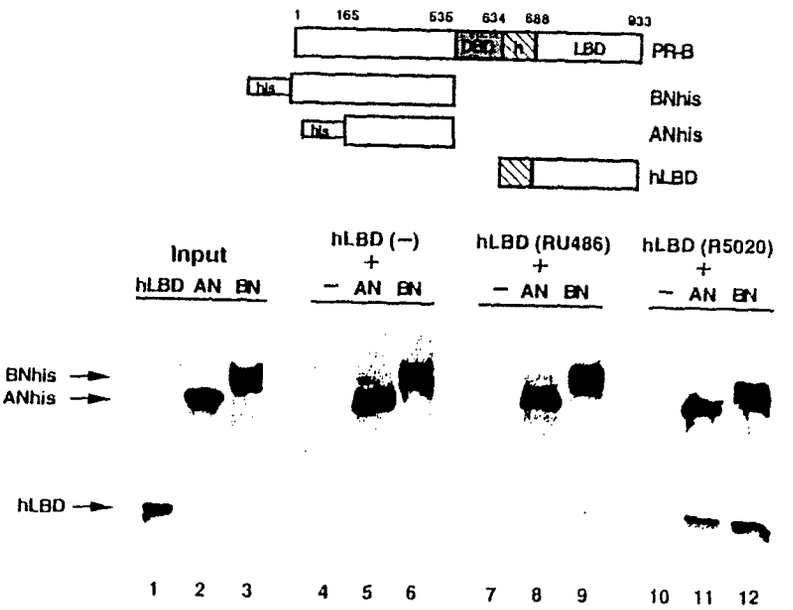

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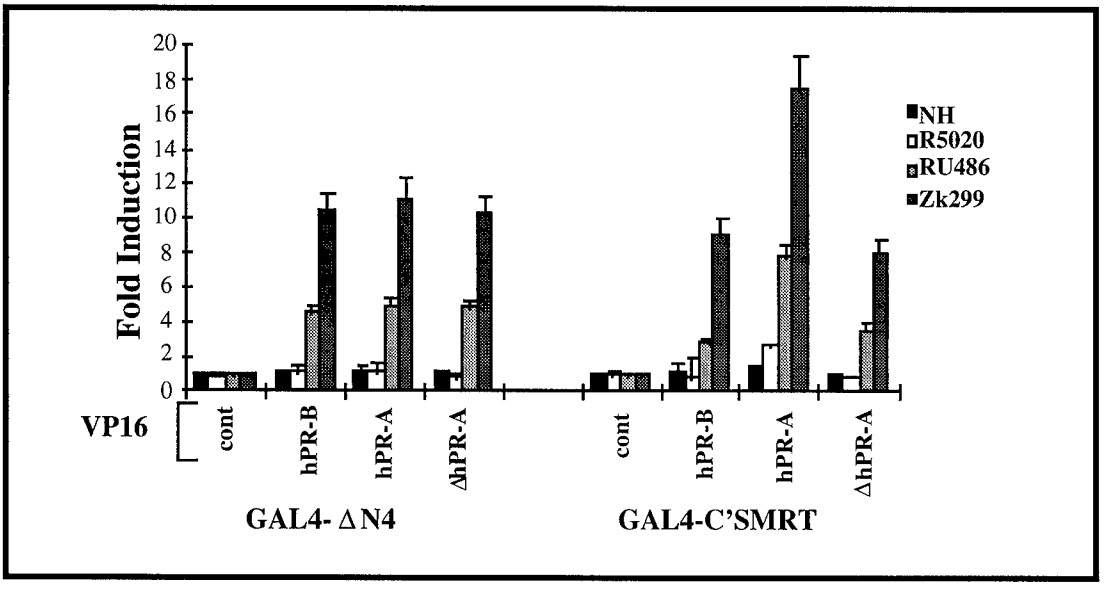

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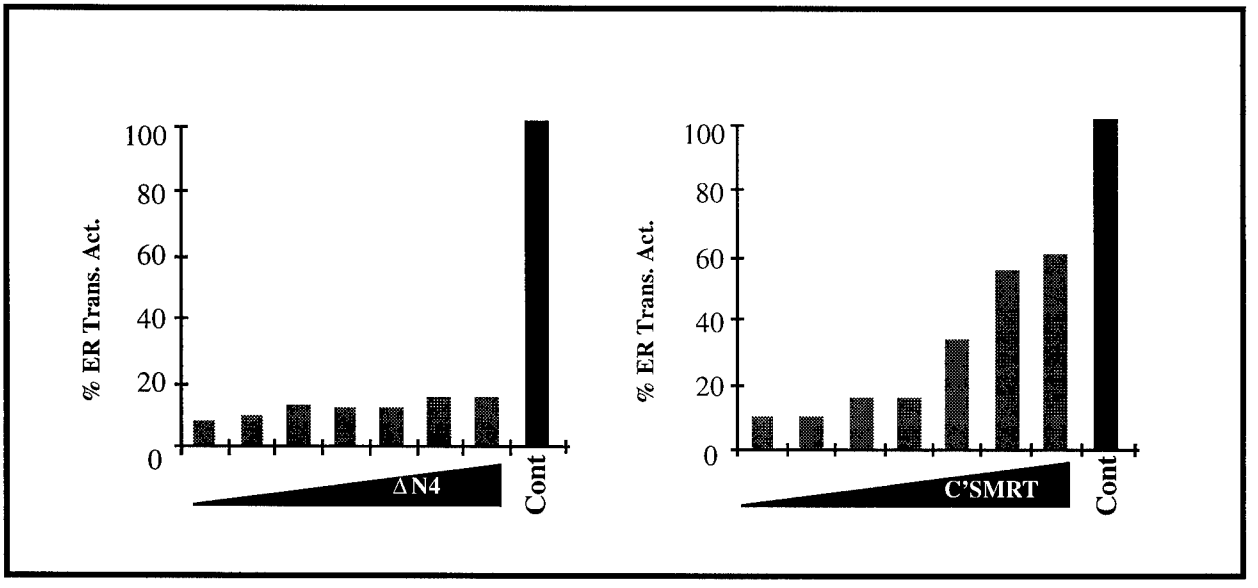

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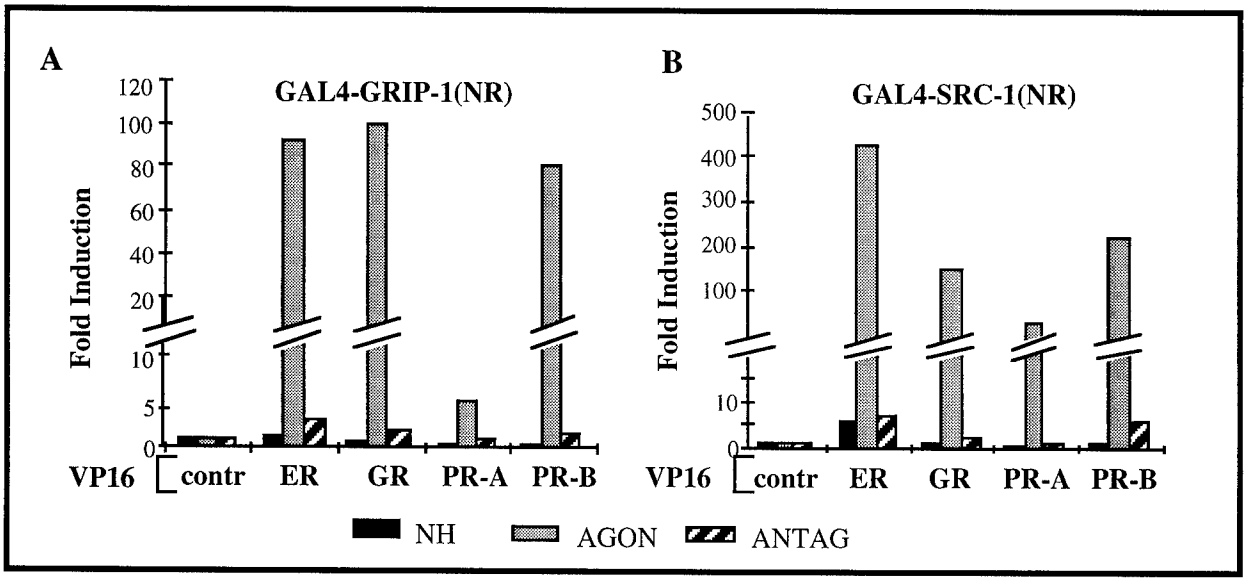

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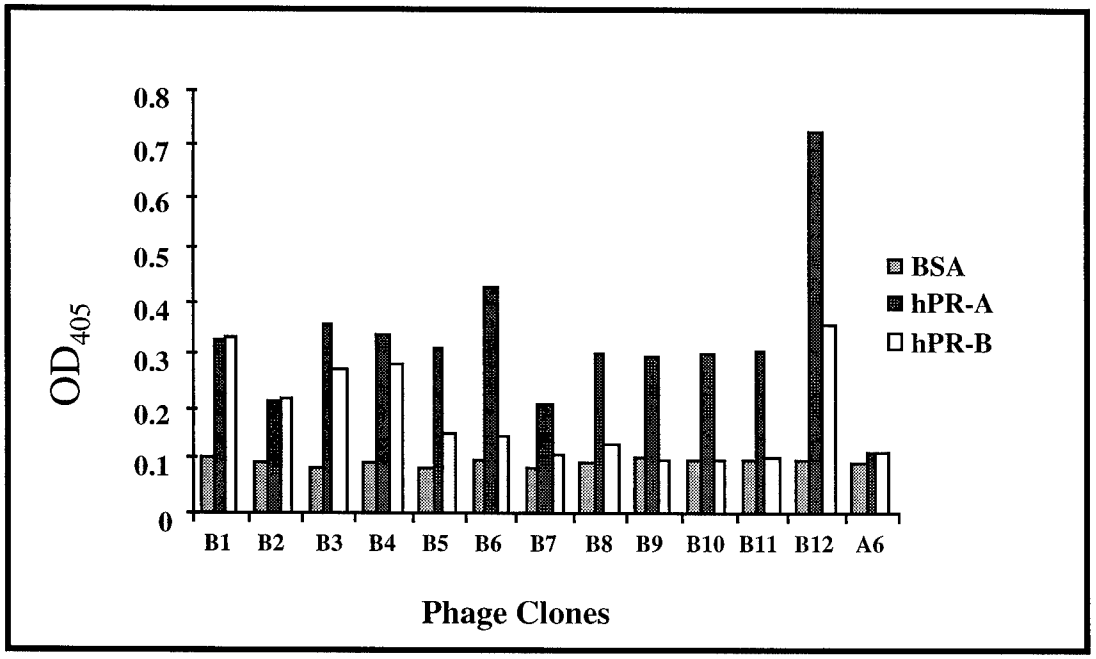

Figure 8

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APPENDIX

1) Research Accomplishments

- Defined the minimal domain of hPR-A required for transdominant repression of ER transcriptional activity
- Showed that the amino termini of hPR-A and hPR-B interact differentially with the carboxyl terminus of PR (hLBD) implying different receptor conformations.
- Showed that the two progesterone receptors exhibit different cofactor interactions.

2) Reportable Outcomes

Manuscripts

Giangrande, P.H. and McDonnell, D.P. (1999). The Opposing Activities of the Two Isoforms of the Human Progesterone Receptor Are Due to Differential Cofactor Binding. (in preparation).

***Tetel, M.J., Giangrande, P.H.**, Leonhardt, S.A., McDonnell, D.P., and Edwards, D.P. (1999). Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor *in vitro* and *in vivo*. *Mol. Endocrinol.* **13**:910-924. *(Co-first authors)

Wagner, B.L., Pollio, G., **Giangrande, P.H.**, Webster, J.C., Breslin, M., Mais, D.E., Cook, C.E., Vedeckis, W.V., Ciblowski, J.A., McDonnell, D.P. (1999). The Novel Progesterone Receptor Antagonists RTI3021-012 and RTI3021-022 Exhibit Complex Glucocorticoid Receptor Antagonist Activities: Implications for the Development of Dissociated Antiprogestins. *Endocrinol.*, **140**: 1449-1458.

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Giangrande, P.H.; Pollio, G.; and McDonnell, D.P. (1997). Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. *J. Biol. Chem.*, **272**: 32889-32900.

Conference Presentations and Posters

Giangrande, P.H. and McDonnell, D.P. The Opposing Activities of the Two Isoforms of the Human Progesterone Receptor Can Be Explained In Part by Differential Cofactor Binding. American Association for Cancer Research, Steroid Hormone Receptors Symposium, Palm Springs, CA, 1999. (Travel Award)

Giangrande, P.H., and McDonnell, D.P. The Differential Activity of the Two Isoforms of the Human Progesterone Receptor Can Be Explained In Part by Differential Co-repressor Binding. Duke University Graduate Student Symposium 1998. (Talk)

Giangrande, P.H., and McDonnell, D.P. The Differential Activity of the Two Isoforms of the Human Progesterone Receptor Can Be Explained In Part by Differential Co-repressor Binding. Keystone Symposia, Lake Tahoe, NV; 1998.

Giangrande, P.H., and McDonnell, D.P. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. Duke University Graduate Student Symposium 1997.

3) Copy of Cited Manuscripts and Abstracts (Attached)

American Association for Cancer Research, Steroid Hormone Receptors Symposium, Palm Springs, CA, 1999.

The Opposing Activities of the Two Isoforms of the Human Progesterone Receptor Can Be Explained In Part by Differential Cofactor Binding.

Paloma H. Giangrande and Donald P. McDonnell . Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA.

In humans, the biological response to progesterone is mediated by two specific, high affinity nuclear receptors which are differentially expressed in target tissues. Both forms of the progesterone receptor (PR), hPR-A (94kDa) and hPR-B (114kDa), are derived from the same gene by alternative initiation of transcription. The only difference between the two receptor isoforms is that the first 164 amino acids of hPR-B are absent from hPR-A. These receptors are functionally different and have distinct roles in progesterone signaling. Specifically, we and others have observed that hPR-B functions as a transcriptional activator in response to agonist stimulation in all cell and promoter contexts examined. This is in contrast to hPR-A which is a transcriptional repressor and functions as a ligand-dependent transdominant repressor of hPR-B transcriptional activity. Of particular importance was the finding that ligand activated hPR-A can also inhibit the transcriptional activity of the estrogen (ER), androgen, glucocorticoid and mineralocorticoid receptors. Thus, hPR-A serves as a point of cross talk between the progesterone-signaling pathway and those regulated by other steroid hormones. The existence of two forms of PR has been documented in most species though the relationship between these receptors remains to be determined in most cases. Analysis of the properties of the chicken progesterone receptors (cPR) however, revealed that both cPR-A and cPR-B were efficient ligand dependent regulators of transcription. This was particularly interesting in view of the high degree of amino acid homology shared between the A-form of the chicken and human PRs. We took advantage of this finding to create a series of chicken/human receptor chimeras, the analysis of which permitted the identification of a specific transcription inhibitory domain located within the first 140 amino acids of hPR-A. Importantly, when transferred to the chicken receptor this inhibitory domain converted cPR-A into a transcriptional repressor. Previously, we have shown that the nuclear co-repressors NCoR and SMRT are important regulators of hPR-B mediated signaling. In the absence of hormone, or in the presence of pure antagonists, it was determined that these co-repressor proteins were able to interact with PR-B. Upon agonist binding however, a conformational change in the receptor occurred which favored the recruitment of co-activator proteins, and the subsequent displacement of co-repressors. These findings, coupled with the identification of an inhibitory domain within hPR-A, suggested that the differences in the transcriptional activity of the two PR-isoforms reflected differences in their ability to interact with co-activators and co-repressors. In support of this hypothesis, we have now shown that both forms of hPR are capable of interacting with SMRT and NCoR. However, the interaction of hPR-A with one of these co-repressors, SMRT, is much stronger than that observed with hPR-B. The physiological significance of this interaction was demonstrated by showing that expression of a dominant negative SMRT variant, cSMRT, reversed hPR-A mediated repression of both hPR-B and hER mediated transcriptional activity. Additionally, using both *in vitro* and *in vivo* methodologies, it was determined that hPR-B, but not hPR-A, interacts efficiently with the co-activators SRC-1 and GRIP. Based on these findings we propose that the ability of hPR-A to function as a transdominant repressor is a product of its enhanced corepressor binding affinity and its reduced affinity for co-activator proteins. Whereas these data clearly explain why hPR-A is not transcriptionally active, it remains to be determined how the hPR-A/SMRT complex can transrepress the transcriptional activity of hPR-B and other steroid hormone receptors.

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The Differential Activity of the Two Isoforms of the Human Progesterone Receptor Can Be Explained In Part by Differential Co-Repressor Binding

Paloma H. Giangrande and Donald P. McDonnell. Department of Pharmacology and Cancer Biology, Molecular Cancer Biology Program, DUMC, Duke University, Durham, NC 27710, USA.

In humans, the biological response to progesterone is mediated by two forms of the progesterone receptor (hPR-A; 94kDa and hPR-B; 114kDa). These two isoforms are transcribed from distinct estrogen-inducible promoters within a single-copy PR gene; the only difference between them is that the first 164 amino acids of hPR-B are absent in hPR-A. In most cell lines, hPR-A functions as a transcriptional repressor of progesterone-responsive promoters, whereas hPR-B functions as a transcriptional activator of the same genes. Interestingly, in these cell contexts, hPR-A also acts as a trans-dominant repressor of the transcriptional activity of other steroid hormone receptors.

In contrast to hPR-A, which functions predominantly as a ligand-dependent transcriptional repressor, we showed that the A isoform of the chicken PR (cPR-A) lacks this trans-dominant repressor function and is a transcriptional activator in all contexts examined. By constructing chimeras between the chicken and human PR we mapped the inhibitory function of hPR-A to the amino terminus of the protein. Although this inhibitory domain is present in hPR-B its activity is only manifested in the context of hPR-A.

The identification of a discrete inhibitory region within hPR-A whose activity is masked in the context of hPR-B, suggests that these two receptor isoforms may interact with different proteins (transcription factors, co-activators, co-repressors) within the cell. In support of this hypothesis, we have shown that the two isoforms of human PR are capable of interacting with the nuclear co-repressor proteins, SMRT and NCoR. Significantly, however, the interaction of hPR-A with the co-repressor SMRT is much stronger than that observed with hPR-B. Interestingly, we show that overexpression of a dominant negative SMRT (C'SMRT), but not a dominant negative NCoR (ΔN4), can reverse hPR-A-mediated transrepression. This important observation suggests that the ability of hPR-A to repress hPR-B transcriptional activity could occur as a consequence of hPR-B/A heterodimerization where the presence of SMRT in the complex prevents transcriptional activation. The observation that hPR-A also inhibits human estrogen receptor transcriptional activity, a receptor with which hPR-A is not able to heterodimerize with, suggests that there must be additional complexity.

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Paloma H. Giangrande and Donald P. McDonnell Department of Pharmacology and Cancer Biology, DUMC, Duke University, Durham, NC 27710, USA.

In humans, the biological response to progesterone is mediated by two distinct forms of the progesterone receptor (hPR-A; 94kD and hPR-B; 114kD). These two isoforms are transcribed from distinct estrogen-inducible promoters within a single-copy PR gene; the only difference between them is that the first 164 amino acids of hPR-B are absent in hPR-A. In most cell lines hPR-A functions as a transcriptional repressor, whereas hPR-B functions as a transcriptional activator of progesterone-responsive genes. Interestingly, in these cell contexts, hPR-A also acts as a trans-dominant repressor of the transcriptional activity of other steroid hormone receptors.

In contrast to hPR-A, which functions predominantly as a ligand dependent transcriptional repressor, we showed that the A isoform of the chicken PR (cPR-A) lacks this trans-dominant repressor function and is a transcriptional activator in all contexts examined. By constructing chimeras between the chicken and human PR we mapped the trans-dominant repressor function of hPR-A to the first 140 amino acids of the protein. Interestingly, this trans-repression function is comprised not only of the "repressor domain" of hPR-A but also requires the context of the receptor in order to function.

The identification of a discrete inhibitory region within hPR-A, which is transferable to another receptor, implies that this region interacts with a set of transcription factors or adaptors which are distinct from those recognized by hPR-B. In support of this hypothesis, we have shown that the two isoforms of human PR are capable of interacting with the nuclear co-repressor proteins, SMRT and NCoR. Significantly, however, the interaction of hPR-A with the co-repressor SMRT is much stronger than that observed with hPR-B. This suggests, therefore, that the amino acid sequences in the amino terminus of hPR-B are important regulators of co-repressor interaction and that differential co-repressor association may explain in part the differential transcriptional activity of hPR-A and hPR-B. The identification of additional cell-specific adaptors will be required in order to better define the mechanism by which hPR-A modulates steroid hormone receptor transcriptional activity.

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Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor.

Paloma H. Giangrande and Donald P. McDonnell. Department of Pharmacology and Cancer Biology, Molecular Cancer Biology Program, DUMC, Duke University, Durham, NC 27710, USA.

In humans, the biological response to progesterone is mediated by two distinct forms of the progesterone receptor (hPR-A; 94Kd and hPR-B; 114Kd). These two isoforms are transcribed from distinct estrogen inducible promoters within a single-copy PR gene; the only difference between them is that the first 164 amino acids of hPR-B are absent in hPR-A. In most cell lines hPR-A functions as a transcriptional repressor, whereas hPR-B functions as a transcriptional activator of progesterone-responsive genes. Interestingly, in these cell contexts, hPR-A also acts as a trans-dominant repressor of the transcriptional activity of other steroid hormone receptors.

In contrast to hPR-A, we have determined that the A isoform of the chicken PR (cPR-A) lacks this trans-dominant repressor function and is a transcriptional activator in all contexts examined. By constructing chimeras between the N-terminal domains of cPR-A and hPR-A we mapped the trans-dominant repressor function of hPR-A to the first 140 amino acids of the protein. Notably, when this "repressor" domain is placed onto cPR-A the activity of the latter changes from a transcriptional activator to a repressor. Interestingly however, this "repressor domain" is necessary, but not sufficient, for trans-repression as it is inactive when it is tethered to a heterologous protein. This suggests that the trans-repression function is comprised not only of the "repressor domain" of hPR-A but also requires the context of the receptor in order to function. The identification of a discrete inhibitory region within hPR-A which is transferable to another receptor implies that this region interacts with a set of transcription co-factors which are distinct from those recognized by hPR-B. The identification of these proteins is a crucial step in the definition of the mechanism by which hPR-A modulates steroid hormone receptor transcriptional activity.

Appendix #1

The Opposing Activities of the Two Isoforms of the Human Progesterone Receptor Are Due to Differential Cofactor Binding

Paloma H. Giangrande and Donald P. McDonnell*

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

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*Address Correspondence to: Dr. Donald P. McDonnell

Department of Pharmacology and Cancer Biology

Box 3813 Duke University Medical Center

Durham, North Carolina 27710

Phone: (919) 684-6035

FAX: (919) 681-7139

E-mail: mcdon016@acpub.duke.edu

ABSTRACT

The human progesterone receptor (PR) exists as two functionally distinct isoforms hPR-A and hPR-B. hPR-B functions as a transcriptional activator in most cell- and promoter-contexts, while hPR-A is transcriptionally inactive and functions as a ligand-dependent transdominant repressor of steroid hormone receptor (SHR) transcriptional activity. Although the precise mechanism of hPR-A-mediated transrepression is not fully understood, an inhibitory domain (ID) within human PR which is necessary for transrepression by hPR-A has been identified. Interestingly, although ID is present within both PR isoforms it is only functionally active in the context of hPR-A, suggesting that hPR-A interacts with a set of cofactors that are distinct from those recognized by hPR-B. In support of this hypothesis, we demonstrate, using a combination of *in vitro* and *in vivo* methodologies, that the two progesterone receptors exhibit different cofactor interactions. Specifically, it was determined that the corepressor SMRT interacts more strongly with hPR-A than with hPR-B, and that this interaction is facilitated by ID. Interestingly, inhibition of SMRT activity using either a dominant negative mutant (C'SMRT) or histone deacetylase inhibitors does not convert hPR-A into a transcriptional activator but it does reverse hPR-A mediated transrepression. Together, these data indicate that the inability of hPR-A to activate transcription and its ability to transrepress SHR transcriptional activity do not occur by the same mechanism. In addition, we observed that hPR-A, unlike hPR-B, was unable to efficiently recruit the transcriptional coactivators GRIP1 and SRC-1 upon agonist binding. Thus, although both receptors contain sequences within their ligand-binding domains known to be required for coactivator binding, the ability to form a productive coactivator interaction is regulated by sequences contained within the amino terminus. We propose that hPR-A is transcriptionally inactive due to its inability to efficiently recruit coactivators. Furthermore, the abilty of hPR-A to transrepress SHR activity requires the transcriptional corepressor SMRT.

INTRODUCTION

The progesterone receptor is a ligand activated transcription factor that belongs to the nuclear receptor superfamily of transcription factors (14). In the absence of hormone, the transcriptionally inactive receptor remains associated with a large complex of heat shock proteins in the nuclei of target cells (49). Upon hormone-binding, the receptor dissociates from the heat-shock protein complex, dimerizes, and binds to progesterone responsive elements (PREs) within the regulatory regions of target genes (4, 33). When bound to DNA, the dimerized receptor contacts components of the general transcription machinery (GTM) either directly (26) or indirectly *via* cofactors such as coactivators and corepressors (41, 47, 56, 19), and either positively or negatively modulates target gene transcription.

Adding to the complexity of its signal transduction pathway is the fact that PR exists in humans as two isoforms, hPR-A (94kDa) and hPR-B (114kDa) (30). hPR-A is a truncated form of hPR-B, lacking the B Upstream Sequence, BUS (aa 1-164). The two isoforms are transcribed from a single gene by alternative initiation of transcription (28, 18). While both forms of PR have similar DNA and ligand-binding affinities (9), they have opposite transcriptional activities (53, 55, 58, 8, 35). In most contexts, hPR-B functions as an activator of progesterone responsive genes, while hPR-A is transcriptionally inactive (53, 55). In addition, hPR-A also functions as a strong transdominant repressor of hPR-B (55) and hER transcriptional activity in the presence of both PR agonists and antagonists (55, 34, 58, 16).

Although the precise mechanism underlying the differential activities of the two human PR isoforms is not fully understood, recent structure function studies of the two receptor isoforms suggest that hPR-B contains three specific activation functions (AFs-1, 2, and 3) whereas hPR-A contains only two. AF-1, located within the amino terminus, and AF-2, in the carboxyl terminus, are common to both hPR-A and hPR-B. The third putative activation function, AF-3, is located within BUS, a region which is absent in hPR-A (44). We believe that AF-3 contributes to hPR-B transcriptional activity by suppressing the activity of an inhibitory domain contained within

sequences common to hPR-A and hPR-B. In support of this, an inhibitory domain (ID) has been identified within the first 140 amino acids of hPR-A which has been shown to prevent hPR-A from functioning as a transcriptional activator, and permit this receptor isoform to function as a transdominant repressor of heterologous steroid receptor transcriptional activity (16). Deletion of the N-terminal 140 amino acids (ID domain) from hPR-A results in a receptor mutant which is functionally indistinguishable from hPR-B (16). Furthermore, Hovland *et al.* have shown that sequences within hPR-A, which contain an ID, inhibit both AF-1 and AF-2 but not AF-3 (23). Cumulatively, these results support the hypothesis that hPR-A, like hPR-B, contains all the sequences necessary for proper transcriptional activation; however hPR-A is transcriptionally inactive because in the absence AF-3, ID prevents AF-1 and/or AF-2 from activating transcription. Thus, it seems that the role of AF-3 is to override this inhibitory function of ID thereby allowing hPR-B to activate transcription (16, 23).

The presence of an inhibitory domain within human PR, whose function is masked in hPR-B, but not in hPR-A, suggests that these two receptor isoforms may interact with different cofactors which could account for their different activities. This hypothesis is further supported by our recent studies which show that the amino termini of hPR-B and hPR-A interact differently with the carboxyl terminus of the PR (hLBD) (51). Specifically, it was shown that the amino terminus of hPR-B, but not that of hPR-A, interacts efficiently with its hLBD both *in vivo* and *in vitro* in an agonist-dependent manner. Thus, the differential interaction between the carboxyl termini and the amino termini of hPR-B and hPR-A may contribute to different cofactor interactions, which in turn may result in differences in the transcriptional activities of the two human PR isoforms.

To investigate potential role(s) of differential cofactor interactions, we examined the ability of hPR-A and hPR-B to associate with different coactivators and corepressors and assessed the effect of these interactions on the receptors' transcriptional activity. We also investigated whether any of these factors could be implicated in hPR-A-mediated transrepression of hER transcriptional activity. From these analyses, we found that antagonist-bound hPR-A interacts more efficiently with the corepressor SMRT than does antagonist-bound hPR-B. The physiological significance of

this interaction was demonstrated using a dominant negative variant of SMRT, C'SMRT, to partially reverse hPR-A transrepression, directly implicating SMRT in the transrepression of hER activity by hPR-A. Furthermore, using both *in vivo* and *in vitro* methodologies, we found that unlike hPR-B, hPR-A did not associate efficiently with coactivators SRC-1 and GRIP1. Thus, the inability of hPR-A, in contrast to hPR-B, to recruit coactivators, as well as its strong association with corepressor proteins, correlates with the differences in the transcriptional activities of the two PR isoforms.

MATERIALS AND METHODS

Biochemicals. DNA restriction and modification enzymes were obtained from Promega (Madison, WI), Boehringer Mannheim, or New England Biolabs (Beverly, MA). PCR reagents were obtained from Perkin-Elmer or Promega (Madison, WI). 17-β-estradiol, dexamethasone, and Trichostatin A (TSA) were purchased from Sigma (St. Louis, MO). R5020 (promegestone) was purchased from NEN Life Science Products. RU486 was a gift from Ligand Pharmaceuticals (San Diego, CA). ZK98299 was a gift from Schering Pharmaceuticals (Berlin, Germany). Secondary antibodies, Hybond-C Extra (nitrocellulose) transfer membrane, and developing film were obtained from Amersham (Arlington Heights, IL). A polyclonal antibody raised against hPR-A was a gift from Nancy Weigel (Baylor College of Medicine, Houston, TX).

Plasmids. pRST7-ERα and SV40-hPR-B were provided by Ligand Pharmaceuticals (San Diego, CA) (10); the expression vectors pBKC-hPR-A and pBKC-hPR-B were reported elsewhere (16); pBKC-Rev-TUP1 and pBKC-βgal have been previously described (57, 32).

The mammalian two-hybrid plasmid pCMX-GAL4-C'SMRT was a gift from J. D. Chen, (University of Massachusetts, Worcester, MA), GAL4N-RIP13ΔN4 was provided by D. D. Moore (Baylor College of Medicine, Houston, TX), and pBKC-DBD was described previously (16). pM, containing the yeast Gal4 DNA binding domain, was purchased from Clontech (San Francisco, CA). pM-GRIP1(NR) was constructed as follows: a PCR-generated fragment from pCMV.HA/GRIP1 (provided by M. Stallcup, University of Southern California, Los Angeles, CA) was subcloned into pM previously digested with *EcoRI* and *BamHI*. The sequences of the oligonucleotides for PCR are: 5'-ggggaattccacagccggctgcatgacagc (forward) and 5'-cgcggatccttccggtaaaccaatatc (reverse). pM-SRC-1(NR) was constructed by digesting pM with *EcoRI* and *BamHI* and subsequent subcloning of a PCR-generated fragment from pCMX-SRC-1 (provided by B. O'Malley, Baylor College of Medicine, Houston, TX). The sequences of the oligonucleotides used to generate the PCR product are: 5'-ccggaattcccgggagacagtaaatactct (forward) and 5'-cgcggatcccaggtttggagttgatct (reverse). The mammalian two-hybrid plasmids

pVP16 and pVP16-T, were purchased from Clontech (San Francisco, CA); the VP16 fusion constructs pVP16-ER, pVP16-GR, pVP16-hPR-A, pVP16-hPR-B, were provided by Ligand Pharmaceuticals (San Diego, CA). pVP16-ΔhPR-A was constructed by digesting the ΔhPR-A fragment from pBKC-ΔhPR-A (16) with *EcoRI* and *BamHI* and subsequent cloning into pVP16 previously digested with *EcoRI* and *BamHI*. All PCR-based cloning was verified by sequencing to assess the fidelity of the resulting constructs.

The GST-fusion plasmid pGEX2TA-C'SMRT was provided by J. D. Chen (University of Massachusetts, Worcester, MA); pGEX-5X-1 was obtained from Pharmacia Biotech (Uppsala, Sweden); pGEX.1-GRIP1 was provided by M. Stallcup (University of Southern California, Los Angeles, CA). The GST fusion plasmid pGEX-5X-1-SRC-1(NR) was constructed as follows: the SRC-1(NR) fragment was digested from pM-SRC-1(NR) with *EcoRI* and *SalI* and subcloned into pGEX-5X-1 previously digested with *EcoRI* and *SalI*. pT7-hPR-A and pT7-hPR-B for *in vitro* translating hPR-A and hPR-B, respectively, were kindly provided by D. P. Edwards (University of Colorado Health Science Center, Denver, CO).

The reporter 5X-GAL4-TATA-LUC, a gift from X.-F. Wang (Duke University Medical Center, Durham, NC), contains five palindromic 17-base pair GAL4-recognition sites cloned into pGL2-TATA-Inr (Stratagene, La Jolla, CA). 2XPRE-TK-LUC contains two copies of a consensus PRE upstream of the thymidine kinase promoter; 3XERE-TATA-LUC contains three copies of vitellogenin ERE cloned into pGL2-TATA-Inr (Stratagene, La Jolla, CA).

Mammalian Transfection and Luciferase Assays. HeLa and HepG2 cells were maintained in modified Eagle's medium containing 10% fetal calf serum (Life Technologies, Grand Island, NY). The cells were plated in 24-well plates (coated with 0.1% gelatin for HepG2 cells) 24 hours prior to lipofectin-mediated transfection as described previously (40). Cells were transfected using a total of 3 μg of DNA per well. After 3 to 5 hours incubation with DNA-lipofectin mixture, the cells were washed and incubated with phenol-red-free media supplemented with 10% charcoal-stripped fetal calf serum and the appropriate ligand and/or TSA treatment for 24 hours. Luciferase and β-galactosidase assays were performed as described previously (40).

***In vitro* Interaction Studies.** [^{35}S]methionine-labeled hPR-A and hPR-B were synthesized using a coupled *in vitro* transcription and translation system in accordance with the manufacturer's protocol (Promega, Madison, WI). The resultant labeled proteins were incubated for 24 hours at 4°C in the presence of either glutathione-S-transferase (GST)-Sepharose, GST-C'SMRT-Sepharose, GST-GRIP1-Sepharose, or GST-SRC-1(NR)-Sepharose, in NETN-A buffer (50 mM NaCl, 20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% Nonidet P40). Following incubation, the beads were washed with NENT-B buffer (100 mM NaCl, 20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% Nonidet P40), and bound proteins were eluted in sample buffer and analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis. The recombinant GST fusion proteins used for the *in vitro* pulldown experiments were produced in *Escherichia coli.* Specifically, the *E. coli* strain BL21 was transformed with either pGEX2TA-C'SMRT, pGEX.1-GRIP1, pGEX-5X-1-SRC-1(NR), or pGEX-5X-1, and grown to an A_{600} of 2.0, after which 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added. Following a 2 hour incubation, the cells were harvested, lysed by sonication, and incubated with glutathione-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) in phosphate buffered saline (PBS) containing 1% Triton X-100. The beads were subsequently washed, resuspended in PBS, and used for the *in vitro* interaction studies.

RESULTS

hPR-A interacts strongly with the corepressor SMRT both *in vivo* and *in vitro*. Data from recent studies suggest that the opposing activities of the two isoforms of human PR may be due to the ability of the two receptors to interact with different cofactors within the cell (16, 23). To determine whether hPR-A and hPR-B bind to different cofactors we assessed the ability of hPR-A and hPR-B to interact with various corepressors and coactivators using both *in vivo* and *in vitro* binding assays.

Recently, we have shown that the nuclear corepressors NCoR and SMRT interact tightly with antagonist-bound hPR-B and less efficiently with mixed-agonist- or agonist-occupied hPR-B (57). To test whether there is a difference between the ability of hPR-A and hPR-B to interact with the corepressors we carried out a series of *in vivo* and *in vitro* binding studies to assess the ability of hPR-A and hPR-B to interact with NCoR or SMRT in the presence of different ligands. The ability of hPR-A to interact *in vivo* with SMRT or NCoR was tested using a mammalian two-hybrid system (57). Specifically, we assessed the ability of full-length hPR-A or hPR-B, fused to the heterologous VP16 acidic activation domain, to interact with either the nuclear receptor interacting domains of NCoR (ΔN4; aa 2002-2453) or SMRT (C'SMRT; aa 981-1495) fused to the GAL4-DNA binding domain (Fig. 1A). Interaction between the two isoforms of PR and the corepressors NCoR or SMRT were assayed by measuring the ability of VP16-hPR-A or VP16-hPR-B fusions to activate transcription from a GAL4-responsive reporter plasmid (5XGAL4-TATA-LUC) in the presence of different PR ligands. Consistent with our previous report, hPR-B interacted with both ΔN4 and C'SMRT in the presence of RU486 and ZK98299 but not in the presence of agonist (R5020), or in the absence of ligand (NH), and the interaction between hPR-B and the corepressors was stronger in the presence of the pure antagonist ZK98299 (57). Like hPR-B, hPR-A interacted with ΔN4 and C'SMRT in the presence of antagonist, but not in the absence of PR ligands. However, the interaction of hPR-A with C'SMRT was stronger than that of hPR-B with C'SMRT (8-fold vs. 3-fold induction of luciferase activity in the presence of

RU486; 17-fold vs. 9-fold in the presence of ZK98299). Interestingly, hPR-A, but not hPR-B, interacted with C'SMRT even in the presence of the agonist R5020, however, R5020-bound hPR-A did not seem to associate with ΔN4 under these conditions. These results indicate that both hPR-A and hPR-B associate with the corepressors NCoR and SMRT in the presence of PR antagonists and that antagonist-bound hPR-A interacts more efficiently with the corepressor SMRT than antagonist-bound hPR-B. Interestingly, ΔhPR-A, the deletion mutant of hPR-A lacking the inhibitory domain (ID) (16), does not interact with C'SMRT as efficiently as the full-length receptor (3.5-fold vs. 7 fold in the presence of RU486; 7-fold vs. 17-fold in the presence of ZK98299) (Fig. 1A). These observations suggest that in the context of hPR-A, ID facilitates binding to SMRT. The VP16-ID fusion alone does not interact with GAL4-C'SMRT (data not shown), suggesting that ID is not sufficient for the interaction of hPR-A with SMRT. The differences in the interactions of the various VP16 fusion proteins were not due to differences in protein expression since all VP16 fusion constructs were shown to express at similar levels by western immunoblot analysis (data not shown).

To determine whether the interaction of hPR-A with SMRT was direct, we carried out an *in vitro* binding analysis (Fig. 1B). In this experiment, the ability of ^{35}S-labeled hPR-A or hPR-B to interact with either bacterially expressed GST alone or a GST-C'SMRT fusion protein was assessed. These studies revealed a specific, robust interaction between hPR-A and C'SMRT in the presence of the antagonist RU486. Not surprisingly, as observed with the mammalian two-hybrid assay, we detected a modest interaction between hPR-A and SMRT even in the presence of agonist R5020 (Fig. 1B). As previously reported, hPR-B also interacts with C'SMRT, albeit in a ligand-independent manner (57). In agreement with the mammalian two-hybrid assay, *in vitro* translated ΔhPR-A did not interact efficiently with GST-C'SMRT under any ligand treatment condition (data not shown). In conclusion, these *in vitro* data correlate with the mammalian two-hybrid data shown in Fig. 1A and suggest that the *in vivo* associations between the receptors and SMRT are direct.

Inactivation of the nuclear receptor silencer, SMRT, does not convert hPR-A into a transcriptional activator. The transcriptional silencers, NCoR and SMRT, have been shown to exist in a complex with the repressor mSin3 and the histone deacetylase HD-1 (also known as HDAC1) suggesting that corepressors mediate gene repression by acting as bridging factors between the receptor and histone deacetylases, thus recruiting HDs to the receptor-DNA complex (1, 20, 39). In Fig. 1 we showed that hPR-A forms a strong association with the corepressor SMRT, implying that hPR-A recruits a repressor complex, composed of SMRT and histone deacetylases, to the promoters of target genes, thereby repressing transcription of target genes. To test whether a complex of SMRT and histone deacetylases with hPR-A was responsible for the inability of hPR-A to activate transcription, we studied the effect of 1) overexpressing a dominant negative variant of SMRT, C'SMRT (Fig. 2A), and 2) increasing concentrations of the deacetylase inhibitor trichostatin A, TSA (Fig. 2B), on hPR-A-mediated transcription.

To test whether hPR-A's association with SMRT was responsible for hPR-A's inability to successfully activate progesterone-responsive promoters, we transiently transfected HeLa cells with an expression vector for hPR-A or hPR-B alone, or in the presence of increasing concentrations of C'SMRT (SMRT dominant negative), together with a 2XPRE-TK-LUC reporter construct. Transcriptional activity was then assessed following stimulation with 10^{-7} M R5020 (Fig. 2A). A control transfection, to assess the basal transcriptional activity of the reporter in the absence of receptors, was included and the value was set to 100%. Clearly, hPR-B-mediated transcriptional activity in the presence of ligand was not significantly affected by increasing C'SMRT concentrations. In contrast, agonist-activated hPR-A repressed basal promoter activity by 64% in the absence of C'SMRT and increasing concentrations of C'SMRT completely reversed hPR-A-mediated repression of basal activity, in a dose dependent manner. This C'SMRT-mediated derepression of basal activity in the presence of agonist-activated hPR-A suggests that when bound to agonist, hPR-A is associated with the corepressor SMRT. Interestingly, even at the highest concentration of C'SMRT used, agonist-activated hPR-A was still not capable of activating transcription beyond the basal level of the reporter in the absence of receptor (Fig. 2A).

To test whether histone deacetylases were involved in hPR-A repression of progesterone responsive promoters, we transiently transfected HeLa cells with an expression vector for hPR-A or hPR-B together with a 2XPRE-TK-LUC reporter construct and induced with 10^{-7} M R5020 alone or in the presence of increasing concentrations of the deacetylase inhibitor, TSA (Fig. 2B). hPR-B-mediated transcriptional activity in the presence of ligand was not affected by increasing TSA concentrations. The basal activity of the 2XPRE-TK promoter was repressed (63%) by agonist-activated hPR-A as observed in Fig. 2A. Increasing concentrations of TSA reversed hPR-A-mediated repression of basal activity, in a dose dependent manner. The increase in basal activity upon TSA treatment suggests that histone deacetylases play a role in repression of basal transcription of progesterone responsive promoters by hPR-A. Not surprisingly, even at the highest concentration of TSA used, hPR-A was not capable of activating transcription from the 2XPRE-TK promoter above the inherent basal level (Fig. 2B). Together, these studies suggest that inhibition of corepressor function is not sufficient to convert hPR-A into a transcriptional activator. In addition, however, it does demonstrate that agonist activated hPR-A can suppress basal transcription.

hPR-B, but not hPR-A, interacts efficiently with the nuclear receptor interacting domains (NR boxes) of the coactivator proteins GRIP1 and SRC-1. The inability of agonist-bound hPR-A to activate transcription in the presence of increasing concentrations of C'SMRT and TSA (Fig. 2A and B) suggests that unlike hPR-B, hPR-A fails to effectively recruit coactivators. Therefore, hPR-B's ability to associate with coactivator proteins and displace corepressors results in an increase in PR transcriptional activity. Conversely, we propose that even when bound to agonist, hPR-A fails to efficiently recruit coactivators and thus is unable to displace corepressors.

To test for an association between hPR-A, hPR-B, and coactivator proteins, we utilized the mammalian two-hybrid system. Specifically, we looked at the ability of full-length hPR-A or hPR-B fused to the heterologous VP16 acidic activation domain, to interact with either the nuclear receptor interacting domains of GRIP1 (GRIP1(NR)) or with the nuclear receptor interacting

domains of SRC-1 (SRC-1(NR)) fused to the GAL4-DNA binding domain (Fig. 3A). Interaction between the two isoforms of PR and the coactivators GRIP1 and SRC-1 respectively, was assayed by measuring the ability of VP16-hPR-A or VP16-hPR-B fusions to activate transcription from a GAL4-responsive reporter plasmid (5XGAL4-TATA-LUC) in the presence of different PR ligands and in the presence of either GAL4-SRC-1(NR) or GAL4-GRIP1(NR). VP16-ER and VP16-GR fusion proteins were used as positive controls. As expected, ER interacts with both GRIP1(NR) and SRC-1(NR) in the presence of estradiol, but not in the absence of ligands or in the presence of antagonists. Similarly, GR interacts with both GRIP1(NR) and SRC-1(NR) in an agonist dependent manner. hPR-B interacts with both SRC-1(NR) and GRIP1(NR) in the presence of R5020 (245-fold and 85-fold over control) but not in the presence of antagonist (RU486) or in the absence of ligand (NH). Interestingly, R5020-bound hPR-A forms a weaker association with both SRC-1(NR) and GRIP1(NR) (45-fold and 9-fold over control) than hPR-B. Cumulatively, the mammalian two-hybrid data suggest that the ability of hPR-B and hPR-A to interact with coactivators correlates with the transcriptional activity of both receptors.

To determine whether the association of hPR-B and hPR-A with the coactivators GRIP1 and SRC-1 was direct, we carried out an *in vitro* binding analysis (Fig. 3B). In this experiment, the ability of ^{35}S-labeled hPR-A or ^{35}S-labeled hPR-B to interact with either bacterially expressed GST alone, GST-GRIP1(NR) (top panel), or GST-SRC-1(NR) (bottom panel), was assessed. These studies revealed a specific, interaction between hPR-B and both GRIP1(NR) and SRC-1(NR) in the presence of R5020 but not in presence of the antagonist RU486 or in the absence of ligands. Interestingly, under the same conditions, hPR-A did not interact with the NR domains of the coactivators GRIP1 and SRC-1. Together, these data indicate that agonist-bound hPR-B but not agonist-bound hPR-A can associate with the nuclear receptor interacting domains of coactivator proteins, implying that the failure of agonist-bound hPR-A to activate transcription may be due to the inability of hPR-A to efficiently recruit coactivators as well as to its inherent higher affinity for corepressor proteins (Fig. 2).

A dominant negative SMRT variant, C'SMRT, can partially reverse hPR-A mediated repression of hER transcriptional activity. Recently, we showed that removal of ID from the A-isoform of PR causes hPR-A to lose its ability to transrepress heterologous steroid hormone receptor transcriptional activity and permit it to function as a transcriptional activator when assayed on a progesterone-responsive promoter (16). This observation, together with the mammalian two-hybrid data (Fig. 1A) suggests that ID, in the context of hPR-A allows human PR to acquire a conformation that is optimal for corepressor binding and/or ID is one of the corepressor binding sites present in PR. Cumulatively, these observations suggest that the inability of hPR-A to activate transcription and its ability to transrepress ER-mediated activity are related and may involve the corepressor SMRT.

To test whether SMRT is also involved in hPR-A-mediated transrepression of hER transcriptional activity we studied the effect of overexpressing the dominant negative C'SMRT on hPR-A-mediated transrepression of hER transcriptional activity. This was accomplished by transiently transfecting HeLa cells with expression vectors for hER, hPR-A, and a 3XERE-TATA-LUC reporter construct, in the presence of increasing amounts of GAL4-C'SMRT or GAL4-ΔN4 (Fig. 4A). In this experiment, increasing amounts of GAL4-ΔN4 had very little effect on hPR-A mediated transrepression in the presence of 10^{-7} M RU486. However, C'SMRT reversed hPR-A-mediated transrepression of ER activity in a dose dependent manner (from 13% ER Transcriptional Activity to 60%). Increasing amounts of GAL4-C'SMRT has no effect on estradiol-mediated ER transcriptional activity in the absence of hPR-A (data not shown). To test the effect of various progesterone ligands on the ability of C'SMRT to reverse hPR-A-mediated transrepression, we transfected HeLa cells as described above and induced with 10^{-7} M estradiol alone or in the presence of either 10^{-7} M R5020, 10^{-7} M RU486, or 10^{-5} M ZK98299 (Fig. 4B). In the presence of R5020, C'SMRT reversed hPR-A-mediated transrepression of ER activity in a dose dependent manner (from 25% to 46% ER Transcriptional Activity; white bars). Reversal of hPR-A-mediated transrepression by C'SMRT in the presence of the antagonist RU486 was from 15% to 62% ER Transcriptional Activity (Fig. 3A; light gray bars). Interestingly, in the presence of the pure

antagonist ZK98299, C'SMRT reversed hPR-A-mediated transrepression of hER activity from 16% to about 80% (dark gray bars) (Fig. 4B). These results suggest that C'SMRT is better at reversing hPR-A-mediated transrepression when the receptor is occupied by antagonist than by agonists. This observation correlates with the mammalian two-hybrid data which indicates that the interaction of hPR-A with GAL4-C'SMRT is greater in the presence of antiprogestins (Fig. 1).

The deacetylase inhibitor, trichostatin A (TSA), partially reverses hPR-A mediated transrepression of hER transcriptional activity. To further assess the involvement of a corepressor complex in hPR-A mediated transrepression of ER transcriptional activity, we examined whether the deacetylase inhibitor TSA could reverse hPR-A-mediated transrepression (Fig. 5). We transiently transfected HeLa cells with expression constructs for hER and either hPR-A or a control plasmid together with a 3XERE-TATA-LUC reporter construct in the presence of 10^{-7} M estradiol and 10^{-7} M RU486 alone or together with increasing concentrations of the deacetylase inhibitor TSA. Estradiol-dependent activation of the 3XERE-TATA promoter in HeLa cells expressing hER together with control plasmid was not significantly affected by coaddition of increasing concentrations of TSA (data not shown). In this experiment TSA is capable of partially reversing hPR-A transrepression of ER activity in a dose dependent manner. In conclusion, the experiments detailed above suggest that the strong interaction of hPR-A with the SMRT corepressor complex might be responsible for the inability of hPR-A to activate transcription, as well as its ability to act as a potent transrepressor of heterologous steroid hormone receptor transcriptional activity.

Antagonist-bound hPR-A is a stronger transrepressor of hER transcriptional activity than agonist-bound hPR-A. Previously, we have shown that in the presence of either agonists or antagonists, hPR-A, but not hPR-B, is capable of transdominant repression of steroid hormone receptor transcriptional activity (55, 35, 58, 16). If SMRT is involved in hPR-A transrepression of ER-mediated transcription and SMRT interacts more strongly with antagonist/hPR-A than with agonist/hPR-A (Fig. 1), then it follows that hPR-A should be a more potent transrepressor of ER activity when bound to antagonist than agonist.

To determine whether there was a difference in the ability of hPR-A to transrepress steroid hormone receptor transcriptional activity in the presence of different progestins, we performed transient transfection assays as previously reported (16). Specifically, we transfected HeLa cells with expression vectors for hER, and either hPR-A or control vector, together with a 3XERE-TATA-LUC reporter construct (Fig. 6) Following transfection, the cells were induced with 10^{-7} M 17-β-estradiol alone or together with increasing concentrations (from 10^{-12} M to 10^{-5} M) of either agonist (R5020), type I antagonist (RU486), or type II antagonist (ZK98299). As reported previously, hPR-A in the presence of 10^{-7} M R5020 transrepressed hER activity by 70% (16). Interestingly, antagonist-bound hPR-A was a slightly more potent transrepressor of hER transcriptional activity (87% in the presence of 10^{-7} M RU486). ZK98299-bound hPR-A was also capable of transrepressing hER transcriptional activity (93% at 10^{-6} M ZK98299). ZK98299-bound hPR-A was a better transrepressor than either R5020- or RU486-bound hPR-A at high ZK98299 (10^{-6} M to 10^{-5} M) concentrations. This effect of ZK98299 is consistent with the fact that higher concentrations of ZK98299 are required to exhibit the same level of antagonist activity as that observed with lower concentrations of RU486 (13). Together, these data indicate that while both agonist and antagonist-bound hPR-A transrepress ER-mediated transcription, antagonist-bound hPR-A is a slightly more potent transrepressor than agonist-bound hPR-A.

DISCUSSION

The precise mechanism underlying the opposing transcriptional activities of the two human PR isoforms has intrigued researchers for many years. Recent studies have suggested that when activated by ligand, many nuclear hormone receptors (NHRs) undergo a unique conformational change which allows the receptors to dissociate from a corepressor complex, containing histone deacetylase activity, and recruit a coactivator complex, containing histone acetylase activity, thus, resulting in target gene transcriptional activation (22, 59, 45, 48, 17, 57, 52). Specifically, transcriptional repression was shown to correlate with the ability of the receptors to bind the corepressors, NCoR and SMRT (19, 57). Conversely, transcriptional activation by NHRs was observed to correlate with the recruitment of coactivators to the promoter region of target genes (41, 21, 27, 46, 17). To determine whether the opposing transcriptional activities of hPR-A and hPR-B were due to differential cofactor association we looked at the ability of hPR-A and hPR-B to interact with different coactivators and corepressors and assessed the effect of these associations on the receptors' transcriptional activity. Using both *in vivo* and *in vitro* methodologies we found that antagonist-bound hPR-A interacts more efficiently with the corepressor SMRT than antagonist-bound hPR-B (Fig. 1). The physiological significance of this interaction was demonstrated by the partial reversal of hPR-A-mediated transrepression of ER activity in the presence of a dominant negative form of SMRT (Fig. 4). In addition, we also observed that, unlike hPR-B, hPR-A did not associate efficiently with coactivators SRC-1 and GRIP1 (Fig. 3). Thus, the strong interaction of hPR-A with SMRT along with its inability to efficiently engage coactivators explains why hPR-A is unable to activate target gene transcription.

Initially, it was proposed that the differences in the transcriptional activities of hPR-A and hPR-B were due to a third potential activation function (AF), AF-3, present within the extreme amino terminus of hPR-B, a region which is absent in hPR-A (44). Thus, it was proposed that full transcriptional activity of hPR-B was a result of the functional synergy of the activation functions located in the amino terminus (AF-3 and AF-1) and the carboxyl terminus (AF-2).

However, unlike AF-1 and AF-2, AF-3 does not demonstrate autonomous activity when fused to a heterologous DBD (38, 44), suggesting that instead of functioning as a classical AF, AF-3 might be required for proper AF-1 and AF-2 transcriptional activity. The two ways in which AF-3 may contribute to hPR-B transcriptional activity are: 1) directly, by enhancing the activity of AF-1 or AF-2, or 2) indirectly, by suppressing an inhibitory function contained within sequences common to both hPR-A and hPR-B (28, 16). Evidence in support of the latter hypothesis came from our studies, as well as those of others, which identified an inhibitory domain within the amino terminus of hPR-A which, when deleted, resulted in a receptor mutant functionally indistinguishable from hPR-B (16, 23, 24). Specifically, we demonstrated that the first 140 amino acids of hPR-A are necessary for its ability to function as a transcriptional inhibitor as well as a transrepressor of heterologous steroid receptor transcriptional activity (16). Thus, the role of AF-3 is to override the inhibitory domain present within the amino terminus of the receptor allowing hPR-B to activate transcription (16, 23).

In addition to the human progesterone receptor, several other transcription factors have been shown to contain both activation and repression functions. Examples of such factors include the lymphoid specific transcription factor, Oct-2a (15); members of the AP1 family of transcription factors: c-Fos, c-Jun, and the related protein FosB (2, 3, 7); a member of the basic region-leucine zipper (bZIP)-containing family of transcription factors, ATF-2 (31); RORα, the orphan nuclear receptor which plays a critical role in cerebellar development (19). The repressor domains within these proteins were identified by creating deletions which enhanced their overall transcriptional activity (2, 3, 12, 7, 15, 31, 19). Of particular relevance to our studies of hPR-A, it was shown that the ability of RORα to repress transcription correlated with the ability of the inhibitory domain within RORα to recruit the corepressors NCoR and SMRT *in vitro* (19). In addition, RORα was shown to preferentially associate with NCoR and not SMRT *in vivo*. When we tested the ability of hPR-A and hPR-B to interact with NCoR and SMRT in the presence of antagonist we found that while both receptors associate with NCoR, hPR-A interacts more efficiently with SMRT than hPR-B, both *in vitro* as well as in a mammalian two-hybrid assay (Fig. 1). Furthermore, the deletion

mutant lacking the inhibitory domain, ΔhPR-A, loses the ability to strongly associate with SMRT as observed with the mammalian two-hybrid assay in Fig. 1A. This implies that like RORα, hPR-A requires its inhibitory domain for optimal corepressor interaction. Thus, the presence of an inhibitory domain within nuclear hormone receptors which is necessary for corepressor association might be a common mechanism for transcriptional repression.

Transcriptional activation correlates with dissociation of corepressors and recruitment of coactivators by the agonist-occupied receptor (reviewed in 52). We observed that while R5020-activated hPR-B loses the ability to interact with SMRT *in vivo*, R5020-bound hPR-A still shows a significant association with the corepressor (Fig. 1A), suggesting that agonist-bound hPR-A is not capable of properly dissociating from the corepressors. The functional significance of this interaction is shown in Fig. 2 where R5020-bound hPR-A represses basal transcription of a progesterone-responsive promoter by 63%. Reversal of hPR-A-mediated repression was achieved by using increasing amounts of C'SMRT, as well as of the deacetylase inhibitor TSA, suggesting that in the presence of agonist, hPR-A is still associated with corepressor complexes containing histone deacetylase activity. Interestingly, even at the highest concentration of C'SMRT or TSA, R5020-bound hPR-A, unlike hPR-B, was unable to activate transcription from this promoter above basal levels. This implies that the unique sequences present at the amino terminus of hPR-B are required for proper transcriptional activation.

The role of the amino terminus of the human progesterone receptor in facilitating maximal transcriptional activation by hPR-B is supported by recent studies which have proposed that full transcriptional activation of steroid hormone receptors requires functional synergy between the activation functions located at the carboxyl terminus and those at the amino terminus of the receptors (6, 50, 38, 43, 54, 36). This synergy involves an agonist-dependent association between the amino and carboxyl AFs of ER (29), the androgen receptor (AR) (25, 11, 5), and hPR-A and hPR-B respectively (51). Interestingly, in the case of hPR-A and hPR-B, the amino terminus of hPR-B containing AF-3, was shown to interact more efficiently with the carboxyl terminus of the receptor than the amino terminus of hPR-A lacking AF-3 (51). This agonist-

dependent interaction was enhanced by the addition of SRC-1 and CBP, while dominant negative variants of SRC-1 and CBP respectively, completely abolished this interaction, suggesting that these coactivators may be required for transcriptional synergy between the amino terminal and carboxyl terminal AFs of the receptor (51). The role of coactivators as bridging factors between the amino and carboxyl AFs of receptors is further supported by a previous study which mapped various progesterone receptor interaction sites on SRC-1 thus allowing SRC-1 to act as a bridging factor between the amino (AF-1) and the carboxyl (AF-2) containing domains of the receptor (42).

The more efficient agonist-dependent interaction of the carboxyl terminus with the amino terminus of hPR-B than with the amino terminus of hPR-A, correlates with the ability of hPR-B to activate transcription in the presence of agonist (55, 53, 34, 58, 16, 23, 24). Thus, the ability of hPR-B to function as an activator of transcription could be due to the fact that hPR-B, but not hPR-A, undergoes a conformational change which is conducive to coactivator binding. This hypothesis is supported by our findings, reported within, which show that agonist-bound hPR-B, but not agonist-bound hPR-A, efficiently interacts with the nuclear receptor interacting domains of the coactivators GRIP1 and SRC-1 in a mammalian two-hybrid assay (Fig. 3A). Likewise, the inability of antagonist-occupied hPR-B to activate transcription can be explained by an inefficient association between the amino and carboxyl domains of the receptor which prevents recruitment of coactivators and thus permits transcriptional activation. In support of this, we show that antagonist-occupied hPR-B does not interact with the coactivators GRIP1 and SRC-1 (Fig. 3). In contrast, a previous study has reported that both hPR-A and hPR-B interact with full-length SRC-1 in the presence of agonist (42). However, only hPR-B was shown to interact with the nuclear receptor interacting domains of SRC-1, therefore, it remains to be determined whether full-length hPR-A and hPR-B interact differently with different sites on the coactivator. When comparing the interaction of hPR-A and hPR-B with the coactivators *in vitro* we observed that while hPR-A interacted with GRIP1(NR) and SRC-1(NR) *in vivo*, albeit less efficiently than hPR-B, it did not do so *in vitro* (Fig. 3B). One explanation for the absence of an *in vitro* interaction between hPR-A and the nuclear receptor interacting domains of SRC-1 and GRIP1 is that the interaction of hPR-A

with the coactivators observed with the mammalian two-hybrid assay is dependent on other protein(s) which associate with hPR-A and/or SRC-1(NR) *in vivo* and stabilize this interaction.

Our working models to explain the opposing transcriptional activities of hPR-A and hPR-B is depicted in Fig. 7A. We propose that hPR-B is a transcriptional activator of progesterone responsive promoters since upon binding hormone, hPR-B undergoes a conformational change which allows it to dissociate from corepressor proteins and recruit coactivators. This productive interaction with the coactivators allows the receptor to activate transcription from the promoters of target genes. Conversely, under the same conditions, hPR-A is transcriptionally inactive because, unlike hPR-B, it cannot fully dissociate from corepressors and thus is not effective in recruiting coactivators to the promoters of target genes.

Furthermore, we propose that the silencing mediator SMRT is involved in hPR-A-mediated transrepression of ER-mediated transcription. In our model (Fig. 7B), ER activates transcription by recruiting a coactivator complex to the promoters of target genes. In this scenario, hPR-A/SMRT complex targets and sequesters a member of the ER-coactivator complex thus interfering with ER-mediated transcriptional activity. Conversely, hPR-B is unable to target this factor necessary for proper ER transcriptional activity.

Whereas the data presented within clearly explains why hPR-B acts as a strong transcriptional activator of progesterone responsive promoters and why hPR-A is transcriptionally inactive, it remains to be determined how the hPR-A/SMRT complex can transrepress the transcriptional activity of hPR-B as well as that of other steroid hormone receptors. We believe that the BUS region at the amino terminus of hPR-B allows the activation functions AF-1 and AF-2 within hPR-B to interact with cofactors required for transcriptional activity. The absence of BUS in hPR-A may allow hPR-A to interact with a different set of proteins and form a complex which can interfere with ligand-dependent steroid hormone receptor transcriptional activity. Formal proof of this hypothesis awaits the identification of factors which can distinguish between hPR-A and hPR-B.

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Figure Legends

Figure 1. hPR-A interacts strongly with the corepressor SMRT both *in vivo* and *in vitro*. (**A**) HeLa cells were transiently transfected with 0.5 μg 5X-GAL4-TATA-LUC, 50 ng pBKC-βgal, 1 μg of either pCMX-GAL4-C'SMRT (GAL4-C'SMRT) or GAL4N-RIP13ΔN4 (GAL4-ΔN4), 1 μg of either pVP16-T (control), pVP16-hPR-B, pVP16-hPR-A, or pVP16-ΔhPR-A, and 0.45 μg of pBSII-KS. Transcriptional activity was assayed on the 5XGAL4-TATA-LUC reporter and represents an indirect measure of the binding of the fusion proteins. Transcriptional activity was measured following the addition of agonist (10^{-7} M progesterone) or antagonists (10^{-7} M RU486 and 10^{-7} M ZK98299). A control was done in the absence of ligands (NH). Transfections were normalized for efficiency using an internal β-galactosidase control plasmid (pBKC- βgal). The data are represented as Fold Induction over the control interaction between GAL4-C'SMRT and VP16-T for each ligand treatment group which was normalized to 1.0. Each data point represents the average of triplicate determinations of the transcriptional activity under the given experimental conditions from three separate experiments. (**B**) GST pulldown assay. The fusion protein GST-C'SMRT containing the carboxyl terminus of SMRT fused onto GST was immobilized onto Glutathione beads and incubated at 4°C for 24 hours with *in vitro* translated hPR-A or hPR-B, in the presence of either vehicle (NH), R5020, or RU486. An equimolar amount of GST was used as a negative control for each condition tested.

Figure 2. Inactivation of the nuclear receptor silencer, SMRT, does not convert hPR-A into a transcriptional activator. (**A**) HeLa cells were transiently transfected with 1.5 μg of 2XPRE-TK-LUC, 50 ng pBKC-βgal, either 52 ng of pBKC-hPR-B, 48 ng pBKC-hPR-A, or 46 ng of pBKC-RevTUP1, and increasing concentrations (varying from 0 to 1 μg) of GAL4-C'SMRT, a SMRT dominant negative. Varying amounts of pBKC-DBD was added to balance the amount of input GAL4-DBD. pBSK-II was added to normalize the total DNA to 3 μg. The transcriptional activity of these vectors was assayed on a 2XPRE-TK-LUC reporter and measured after the addition of 10^{-7} M R5020. Transfections were normalized for efficiency as mentioned previously. R5020-mediated transcriptional activity in the presence of increasing concentrations C'SMRT was normalized to NH for each concentration of C'SMRT used. Each data point represents the average of triplicate determinations (±SEM) from two separate experiments (n=2) The control represents basal reporter activity in the presence of control vector and was set to 100% Transcriptional Activity. (**B**) HeLa cells were transiently transfected with 1.5 μg 2XPRE-TK-LUC, 50 ng pBKC-βgal, either 50 ng pBKC-hPR-A or 48 ng of pBKC-Rev-TUP1, and varying amounts of pBSK-II for a total of 3 μg. The transcriptional activity of these constructs was measured following the addition of 10^{-7} M R5020 alone or in combination with increasing concentrations (0, 10^{-8}, 10^{-7}, and 10^{-6} M) of the deacetylase inhibitor Trichostatin A (TSA). Transfections were normalized for efficiency as mentioned above. R5020-mediated transcriptional activity in the presence of increasing concentrations of TSA was normalized to NH for each TSA treatment used. Each data point represents the average of triplicate determinations (±SEM) from two separate experiments (n=2)

Figure 3. hPR-A interacts weakly with the nuclear receptor interacting domains of the coactivator proteins, GRIP1 and SRC-1. (**A**) HeLa cells were transiently transfected with 0.5 μg 5XGAL4-TATA-LUC, 50 ng pBKC-βgal, 1 μg of either pM-GRIP1(NR) or pM-SRC-1(NR), 1 μg of either pVP16-T, pVP16-ER, pVP16-GR, pVP16-hPR-B, or pVP16-hPR-A, and 0.45 μg of pBSII-KS. Transcriptional activity of the luciferase gene was assayed on the 5XGAL4-TATA-LUC reporter as in Fig. 1A. Transcriptional activity was measured following the addition of agonists (10^{-7} M R5020, or 10^{-7} M 17-β-estradiol, or 10^{-7} M dexamethasone) or antagonists (10^{-7} M RU486, or 10^{-7} M ICI182,780). A control was done in the absence of ligands (NH). Transfections were normalized for efficiency as mentioned above. The data are represented as Fold Induction over the control interaction between GAL4-GRIP1(NR) or GAL4-SRC-1(NR) and VP16-T for each ligand treatment group which was normalized to 1.0. Each data point represents the average of triplicate determinations from one representative experiment. The average coefficient of variation at each point was <10%, n=3. (**B**) GST pulldown assay. The fusion proteins GST-GRIP1(NR) (top panel) and GST-SRC-1(NR) (bottom panel) containing the nuclear receptor interacting domains of the coactivators GRIP1 and SRC-1, respectively, fused onto Glutathione-S-Transferase (GST) were immobilized onto Glutathione beads and incubated at 4°C for 24 hours with *in vitro* translated S^{35}-hPR-A or S^{35}-hPR-B, in the presence of vehicle (NH), R5020, or RU486. An equimolar amount of GST alone was used as a negative control for each condition tested.

Figure 4. The dominant negative variant of SMRT, C'SMRT, can partially reverse hPR-A-mediated repression of hER transcriptional activity. (**A**) HeLa cells were transiently transfected with 1 μg of 3XERE-TATA-LUC, 50 pBKC-βgal, 0.45 μg pRST7-ER, 0.3 μg pBKC-hPR-A, and increasing concentrations (ranging from 0 - 1.2 μg) of GAL4-C'SMRT. Varying amounts of pBKC-DBD was added to balance the amount of input GAL4-DBD. Transcriptional activity was assayed on the 3XERE-TATA-LUC reporter. Transcriptional activity was measured 24 hours after the addition of 10^{-7} M RU486 and 10^{-7} M 17-β-estradiol. A control was done in the absence of ligands (not shown). The data are presented as % activation where 100% represents a measure of 17-β-estradiol dependent transactivation by hER in the absence of RU486 (CONT). (**B**) HeLa cells were transiently transfected as in Fig 4A. Transcriptional activity was measured 24 hours after the addition of 10^{-7} M 17-β-estradiol and either 10^{-7} M R5020, 10^{-7} M RU486, or 10^{-5} M ZK98299. A control was done in the absence of ligands (not shown). The data are presented as % activation where 100% represents a measure of 17-β-estradiol dependent transactivation by hER in the absence of progestins or antiprogestins (CONT) for each experimental condition. The average coefficient of variation at each point was <12%. The data from a single representative experiment are shown (n=3).

Figure 5. The deacetylase inhibitor Trichostatin A (TSA) can partially reverse hPR-A transrepression of ER-mediated transcriptional activity. HeLa cells were transiently transfected as mentioned previously with 1.5 μg of 3XERE-TATA-LUC, 50 ng pBKC-βgal, 0.50 μg pRST7-ER, and either 0.481 μg pBKC-hPR-A or 0.467 μg of pBKC-Rev-TUP1. Variable amounts of pBSII-KS were used for a total of 3 μg of DNA. Transcriptional activity of the 3XERE-TATA-LUC reporter was measured 24 hours after the addition of 10^{-7} M 17-β-estradiol and 10^{-7} M RU486 alone or in combination with increasing concentrations of TSA (0, 10^{-8}, 10^{-7}, and 10^{-6} M). A control was done in the absence of ligands (not shown). The data are presented as % activation where 100% represents a measure of 17-β-estradiol dependent transactivation by hER in the absence of RU486 (CONT). The data from one representative experiment are shown, n=2. The average coefficient of variation at each point was <15%.

Figure 6. Antagonist-bound hPR-A functions as a more efficient transrepressor of hER transcriptional activity than agonist-bound hPR-A. HeLa cells were transiently transfected as in Fig 5. The transcriptional activity of the reporter was measured following the addition of 10^{-7} M 17-β-estradiol alone or in combination with increasing concentrations (ranging from 10^{-12} to 10^{-5} M) of either R5020, RU486, ZK98299. The data are calculated as % activation, where 100% represents a measure of 17-β-estradiol activation by hER in the presence of a control vector, pBKC-Rev-TUP1, or in the presence of hPR-A all in the absence of added PR ligands. This value is independently calculated for each data point. % hER transcriptional activity in the presence of hPR-A was normalized to % hER activity obtained in the absence of hPR-A, control, for each ligand treatment group. R5020-bound hPR-A, RU486-bound hPR-A, and ZK98299-bound hPR-A, respectively. Each data point represents the average of triplicate determinations of the transcriptional activity under the given experimental conditions from a single representative experiment (n=2). The average coefficient of variation at each hormone concentration was <10%.

Figure 7. Two distinct models are required to describe the molecular mechanism of action of hPR-A. (**A**) *Transcriptional Activation.* Based on the *in vivo* and the *in vitro* binding studies, we propose that hPR-A interacts more efficiently with corepressors and less efficiently with coactivators than hPR-B. In the presence of hormone, hPR-B, but not hPR-A, undergoes a favorable conformational change which allows it to displace corepressors (CoR) and recruit coactivator proteins (CoA), thus allowing hPR-B to activate transcription from progesterone responsive promoters. (**B**) *Transrepression.* Based on our *in vivo* transrepression data, we propose that hPR-A transrepresses ER-mediated transcription by a *transcriptional interference* mechanism. In this model, ER activates transcription by recruiting a complex of coactivator proteins (ER CoA complex) to the regulatory region of target genes. hPR-A (A), but not hPR-B (B), targets and sequesters a member of the ER CoA complex thus preventing ER from activating transcription. hPR-A transrepression of ER transcriptional activity is further enhanced by the recruitment by hPR-A of the corepressor SMRT (CoR). PRE, Progesterone Responsive Element; ERE, Estrogen Responsive Element.

Hormone-Dependent Interaction between the Amino- and Carboxyl-Terminal Domains of Progesterone Receptor *in Vitro* and *in Vivo*

Marc J. Tetel*†, Paloma H. Giangrande†, Susan A. Leonhardt, Donald P. McDonnell, and Dean P. Edwards

Department of Pathology and Molecular Biology Program (M.J.T., S.A.L., D.P.E.)
University of Colorado Health Sciences Center
Denver, Colorado 80262

Department of Pharmacology and Cancer Biology (P.H.G., D.P.M.)
Duke University Medical Center
Durham, North Carolina 27710

Full transcriptional activation by steroid hormone receptors requires functional synergy between two transcriptional activation domains (AF) located in the amino (AF-1) and carboxyl (AF-2) terminal regions. One possible mechanism for achieving this functional synergy is a physical intramolecular association between amino (N-) and carboxyl (C-) domains of the receptor. Human progesterone receptor (PR) is expressed in two forms that have distinct functional activities: full-length PR-B and the amino-terminally truncated PR-A. PR-B is generally a stronger activator than PR-A, whereas under certain conditions PR-A can act as a repressor in *trans* of other steroid receptors. We have analyzed whether separately expressed N- (PR-A and PR-B) and C-domains [hinge plus ligand-binding domain (hLBD)] of PR can functionally interact within cells by mammalian two-hybrid assay and whether this involves direct protein contact as determined *in vitro* with purified expressed domains of PR. A hormone agonist-dependent interaction between N-domains and the hLBD was observed functionally by mammalian two-hybrid assay and by direct protein-protein interaction assay *in vitro*. With both experimental approaches, N-C domain interactions were not induced by the progestin antagonist RU486. However, in the presence of the progestin agonist R5020, the N-domain of PR-B interacted more efficiently with the hLBD than the N-domain of PR-A. Coexpression of steroid receptor coactivator-1 (SRC-1) and the CREB binding protein (CBP), enhanced functional interaction between N- and C-domains by mammalian two-hybrid assay. However, addition of SRC-1 and CBP *in vitro* had no influence on direct interaction between purified N- and C-domains. These results suggest that the interaction between N- and C-domains of PR is direct and requires a hormone agonist-induced conformational change in the LBD that is not allowed by antagonists. Additionally, coactivators are not required for physical association between the N- and C-domains but are capable of enhancing a functionally productive interaction. In addition, the more efficient interaction of the hLBD with the N-domain of PR-B, compared with that of PR-A, suggests that distinct interactions between N- and C-terminal regions contribute to functional differences between PR-A and PR-B. (Molecular Endocrinology 13:910–924, 1999)

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INTRODUCTION

The human progesterone receptor (PR) is a member of the nuclear receptor superfamily of transcriptional activators that regulates development, differentiation, and homeostasis of various reproductive functions (1, 2). PR is expressed as two distinct molecular forms from a single gene: full-length PR-B and truncated PR-A that lacks the first 164 amino acids of the amino terminus (3). PR, as well as other steroid receptors, has a conserved structural and functional organization that has been well characterized (1, 2). Both forms of PR are identical in their centrally located DNA-binding domain (DBD) and carboxyl (C-) terminal ligand-binding domain (LBD). PR-A and PR-B also contain two independent transcriptional activation domains (AF): a constitutive AF-1 in the amino terminus and a hormone-dependent AF-2 in the LBD (4, 5). A third transcriptional modulatory domain has been defined in the amino (N-) terminal segment unique to PR-B that re-

quires interaction with other regions of the receptor (5, 6). Under certain cell and promoter contexts, PR-B is a stronger transcriptional activator than PR-A (7–10). This difference in activity is most likely due to conformational or other structural differences between the N termini of the two-receptor isoforms (3, 11, 12). Under conditions in which PR-A is not an activator, it can functionally repress the transcriptional activity of other steroid receptors (7–10). While the mechanism for this repression by PR-A is not fully understood, a discrete transcriptional inhibitory region has been identified in human PR-A that may allow it to interact with factors that do not interact with PR-B (11, 12).

Steroid receptors, including PR, are latent transcription factors that are inactive in the absence of hormone and undergo a multistep activation process upon binding ligand. Receptor activation includes the steps of ligand-induced conformational change, dissociation from an inactive oligomeric complex composed of heat shock proteins and immunophilins, dimerization, and binding to specific DNA sequences of steroid-responsive genes to thereby alter rates of gene transcription (2, 13–15). The identification of coactivators that interact directly with a broad range of nuclear receptors in a hormone- and AF-2-dependent manner has provided important insights into the mechanism by which receptor-DNA interaction modulates gene transcription. The p160 family of coactivators and the CREB binding protein (CBP) family of coactivators (16–20) have been shown to enhance the transcriptional activity of nuclear receptors and to be essential for maximal hormonal responses *in vivo* (16, 21–23). Nuclear receptor coactivators appear to act as bridging proteins between the receptor and general transcription factors, thereby facilitating recruitment of the preinitiation complex. Coactivators are also believed to be involved in targeted remodeling of chromatin due to their intrinsic histone acetyltransferase activity (24–27). The coactivators identified so far primarily interact with and mediate the function of AF-2; AF-1-specific coactivators have not been identified. However, the p160 coactivators such as steroid receptor coactivator SRC-1 and glucocorticoid receptor-interacting protein GRIP-1 have been recently shown to directly interact with amino-terminal sequences of PR or ER, albeit less efficiently than they interact with AF-2, and to be capable of mediating coactivation function through the amino terminus (28–31).

Under certain cell and promoter contexts, both AF-1 and AF-2 can function independently. However, under most conditions, functional synergy between AF-1 and AF-2 is required for full transcriptional activity (4, 5, 32–39). Studies with estrogen receptor (ER) and androgen receptor (AR) have suggested that an intramolecular association between the amino- and carboxyl-terminal regions of receptor contributes to the functional synergy between AF-1 and AF-2. In a modified mammalian cell two-hybrid interaction assay, separately expressed amino- and carboxyl-terminal domains of ER were observed to functionally interact *in vivo* in a hormone agonist-dependent manner (40). Using both yeast and mammalian two-hybrid interaction assays, several groups have also observed a hormone-agonist dependent interaction between amino- and carboxyl-terminal domains of AR (30, 41–43). It is not clear from these two-hybrid interaction experiments whether amino-carboxyl domain interactions are direct or indirectly mediated by coactivators or other proteins that associate with either domain of the receptor. Functional interactions in a two-hybrid assay could be the result of either direct or indirect binding. Conflicting results have been reported for the effect of nuclear coactivators on functional interactions between N- and C-domains as detected by two-hybrid assays. It was reported that SRC-1 enhances ER N-C domain interactions (44), both SRC-1 and CBP enhanced interactions between the N- and C-domains of AR, while a truncated form of SRC-1 was observed to inhibit these interactions in AR (30). In another study, the transcriptional intermediary factor TIF-2 had no effect on the functional interaction between the N- and C-domains of AR (43). Direct *in vitro* interaction between purified N- and C-domains of steroid receptors has not been reported.

In the present study we have investigated whether the N- and C-domains of human PR are capable of interacting in a hormone agonist-dependent manner. To resolve the question of whether these interdomain interactions are direct or indirect, they were analyzed by direct protein-protein interaction assays *in vitro* with purified N- and C-domain polypeptides of PR and by a mammalian two-hybrid assay. We also investigated whether the N-domains of the A and B forms of PR interact the same or differently with the C-terminal LBD as a possible contributing factor to the different functional activities of the two receptor forms.

RESULTS

Hormone-Agonist Dependent Interaction *in Vitro* between Amino- and Carboxyl-Terminal Domain Polypeptides of PR

Protein-protein interaction *in vitro* between separately expressed N- and C-domains of PR was analyzed initially by a polyhistidine-tagged protein pull-down assay with the polypeptides shown schematically in Fig. 1. The PR fragments included polyhistidine-tagged N-domains of PR-A (ANhis; aa 165–535) and PR-B (BNhis; aa 1–535) and nontagged C-domains containing either the entire LBD (aa 688–933) or the LBD plus hinge region (hLBD; aa 634–933). It should be noted that the expressed N- and C-domains both lack the DBD and thus share no overlapping sequences (Fig. 1) that might contribute to protein-protein interaction through homodimerization. Because the baculovirus PR domain vectors contain polyhistidine tags, it was necessary to cleave the tag from one

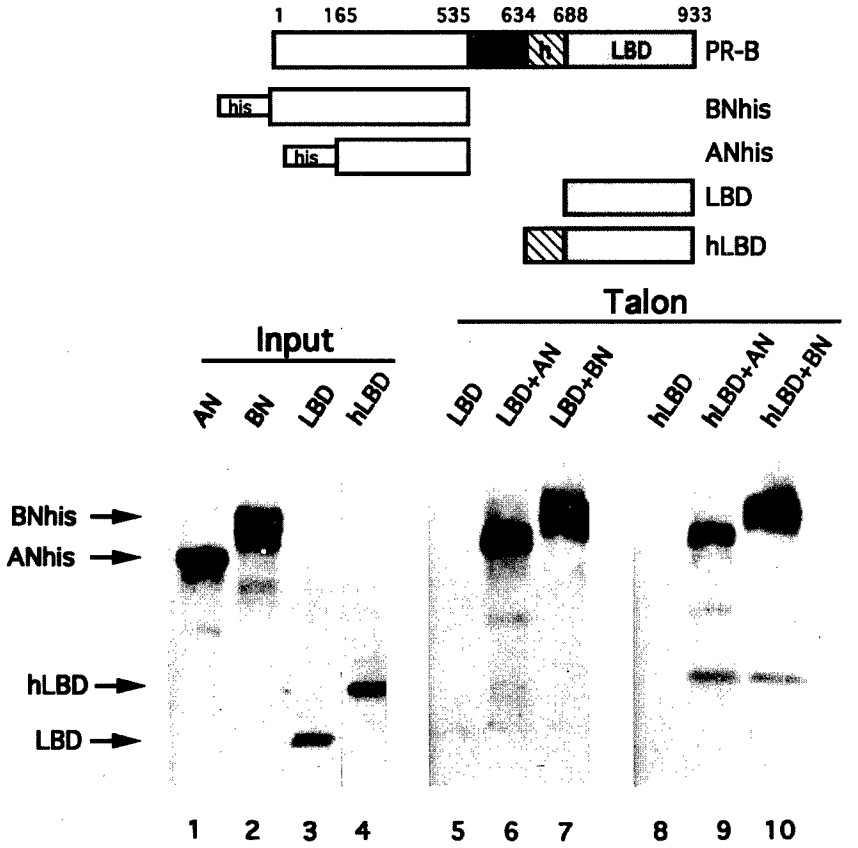

Fig. 1. PR Amino-Carboxyl Terminal Interactions Detected *in Vitro* by Polyhistidine Pull-Down Assay

Schematic of PR domains expressed in baculovirus (*upper panel*): The amino termini of PR-B (BNhis, aa 1–535) and PR-A (ANhis, aa 165–535) were expressed with a 6× polyhistidine tag. The hinge region (h) and ligand binding domain (hLBD, aa 634–933) and the LBD alone (aa 688–933) were expressed and prepared as nonfusion proteins. Full-length PR-B (aa 1–933) is shown for alignment of all the receptor domains. Six sequential N-terminal histidine residues (his). Whole-cell extracts of infected Sf9 cells containing polyhistidine-tagged N-domains of PR-B (BNhis) or PR-A (ANhis) were mixed with equal amounts (determined by Western blot and steroid-binding analysis) of C-terminal domains (LBD or hLBD) and incubated with metal ion affinity resins (Talon). The LBD and hLBD were bound to the synthetic progestin R5020 during expression in Sf9 cells. After washes of the resin, bound proteins were eluted with 2% SDS and analyzed by Western blot with a mixture of MAbs that recognize epitopes in either the N-domain or the LBD of PR (AB-52 and C-262, respectively). Assay input (10%) of polyhistidine-tagged N-domains (ANhis and BNhis), and the carboxyl-terminal, LBD and hLBD, are shown in lanes 1–4. Lanes 5–7 are the LBD incubated with metal resins (Talon) in the absence (nonspecific binding control, lane 5) or presence of ANhis (lane 6) or BNhis (lane 7). Lanes 8–10 are the hLBD incubated with metal resins in the absence (lane 8) or presence of ANhis (lane 9) or BNhis (lane 10). The Western blot detection method was ^{35}S-labeled protein A and autoradiography.

of the paired PR fragments, which was done with the C-domain polypeptides by treatment with enterokinase as described previously (45). Each domain polypeptide was expressed from baculovirus vectors in Sf9 insect cells, and the C-domains were bound to the synthetic progestin R5020 during expression before cell lysis. Whole-cell extracts containing N- or C-domains were mixed and incubated for 30 min at 4 C before immobilization to metal affinity resins (Talon) through the polyhistidine-tagged N-domain polypeptide. After washing the Talon resins with 15 mM imidazole and 100 mM NaCl to remove nonspecific proteins, bound proteins were eluted and analyzed by Western blot with a mixture of monoclonal antibodies (MAbs) that recognize epitopes in the N terminus (AB-52) and the C terminus of PR (C262) (46, 47). To determine the nonspecific binding of C-domain polypeptides that lack polyhistidine tags, the LBD and hLBD were incubated with Talon resins in the absence of the polyhistidine tagged N-domains of PR (Fig. 1, lanes 5 and 8).

By this pulldown assay, no specific association was detected between the LBD and the N-domains of either form of PR (Fig. 1, lanes 5–7). However, a significant amount of hLBD associated in a specific manner with the N-domain of either PR-A or PR-B (Fig. 1, lanes 8–10). It should be noted that the triplet bands of the N-domain of PR-B are due to phosphorylation sites in the unique N terminus of PR-B (48). The ratio of the C-domain polypeptide specifically associated with Talon-immobilized polyhistidine-tagged N-domains was determined by Phosphorimager analysis from multiple pull-down experiments, and the results are

summarized in Table 1. These quantitative analyses confirmed there was no detectable specific interaction between the LBD and the N-domains of PR-A (LBD/ANhis ratio = 0.02 ± 0.01, n = 3) or PR-B (LBD/BNhis ratio = 0.01 ± 0.01, n = 3). In contrast, a substantial amount of the hLBD specifically associated with the N-domains of either PR-A or PR-B. We also observed a significantly higher ratio of hLBD interaction with the N-domain of PR-B (ratio of hLBD/BNhis = 0.27 ± 0.04) than with the N-domain of PR-A (ratio of hLBD/Anhis = 0.14 ± 0.03; $P < 0.05$), (Table 1, R5020 column). Taken together, these results suggest a direct protein interaction between N- and C-domains of PR that requires both the hinge plus LBD as the minimal C-terminal region and that there is a more efficient interaction of the hLBD with the N terminus of PR-B than with the N-domain of PR-A.

To determine whether interaction between N-domains and the hLBD is dependent on ligand binding, similar polyhistidine-tagged protein pull-down experiments were performed with the hLBD prepared in the unliganded state, or bound to R5020 or the progesterone antagonist RU486. The hLBD did not physically associate with the N-domains of PR-A or PR-B in the absence of ligand (Fig. 2, lanes 4–6) or when bound to RU486 (Fig. 2, lanes 7–9). The hLBD efficiently interacted with the N-domains of PR (A or B form) only when bound to R5020 (Fig. 2, lanes 10–12). Results of quantitative analysis by Phosphorimaging of multiple pull-down experiments are summarized in Table 1 and confirm that interaction between the N- and C-domains of PR *in vitro* is dependent on hormone agonist binding to the hLBD and is not allowed by the antagonist RU486.

Interaction between Amino and Carboxyl Domains of PR Is Direct between Purified PR Fragments and Does Not Require Other Proteins

The *in vitro* protein interaction experiments depicted in Figs. 1 and 2 and summarized in Table 1 were performed with PR domain polypeptides present in crude extracts of Sf9 insect cells. To determine whether these interactions are direct or require other proteins, similar pull-down experiments were done using purified PR domain polypeptides. Baculovirus expressed N-domains of PR (ANhis and BNhis) were purified as described in *Materials and Methods* by affinity chromatography on nickel chelation resins using imidazole to elute the proteins under nondenaturing conditions. Because we encountered problems with low yields of purified polyhistidine-tagged hLBD from nickel resin, followed by enterokinase cleavage necessary to generate nontagged hLBD for polyhistidine pull-down assays, we used a baculovirus-expressed glutathione *S*-transferase (GST)-tagged hLBD and GST-pull down assays for experiments with purified PR fragments. The hLBD-GST was bound to R5020 during expression in Sf9 insect cells and was purified by affinity chromatography with glutathione-Sepharose resins as described in *Materials and Methods* using reduced glutathione to elute the hLBD under nondenaturing conditions. Silver-stained SDS-gels and Western blot to confirm the identity of the PR domain polypeptides shows that the N-domains of PR-A (AN) and PR-B (BN) and the GST-hLBD were purified to greater than 90% (Fig. 3).

Approximately equal amounts (determined from silver-stained SDS gels) of purified N-domains and hLBD-GST were mixed together in GST pull-down assays. The hLBD-GST and a baculovirus-expressed GST as a control for nonspecific binding were preimmobilized to glutathione-Sepharose resins. The hLBD-GST, GST, and blank resins were then incubated with purified N-domain polypeptides, and after washing of the resins in buffer with 125 mM NaCl, bound proteins were eluted and analyzed by Western blot. Detection of specifically associated N-domains was by use of a MAb (1294) that recognizes an epitope in the N-terminal region of human PR that is common to both A and B isoforms (Fig. 4). To confirm equal loading and binding of hLBD-GST to the glutathione-Sepharose resins, separate Western blots were performed with the C-262 MAb that recognizes an epitope in the LBD (not shown). A significant fraction of the N-domain of PR-A (AN) (Fig. 4A) and the N-domain of PR-B (BN) (Fig. 4B) specifically associated with hLBD-GST above the little to no binding of the N-domains to GST, or to blank glutathione-Sepharose resins. Quantitative Phosphorimager analysis from multiple

Table 1. Effect of Ligands on PR Amino-Carboxyl Terminal Domain Interactions *in Vitro*

Interacting Domains	Ratio of hLBD to Amino-Terminal Domain (Mean ± SEM)		
	R5020	No Ligand	RU486
hLBD/ANhis	0.14 ± 0.03 (n=10)[a]	0.0 (n=3)	0.01 ± 0.01 (n=3)
hLBD/BNhis	0.27 ± 0.04 (n=7)[a]	0.0 (n=3)	0.02 ± 0.02 (n=3)

Multiple polyhistidine-tagged protein pull-down assays were quantified by determining the ratio of hLBD to polyhistidine tagged N-domains of PR specifically bound to metal ion affinity resins (Talon). Values (mean ± SEM) were measured by direct PhosphorImager scanning of Western blots for radioactivity (bound [^{35}S]Protein A) in the receptor bands. The hLBD was bound to 200 nM R5020, 200 nM RU486, or no ligand for the final 6 h of infection of Sf9 cells before cell lysis. Receptor was also exposed to the appropriate ligand *in vitro* during the polyhistidine-tagged protein pull-down assay.

[a] $P < 0.05$.

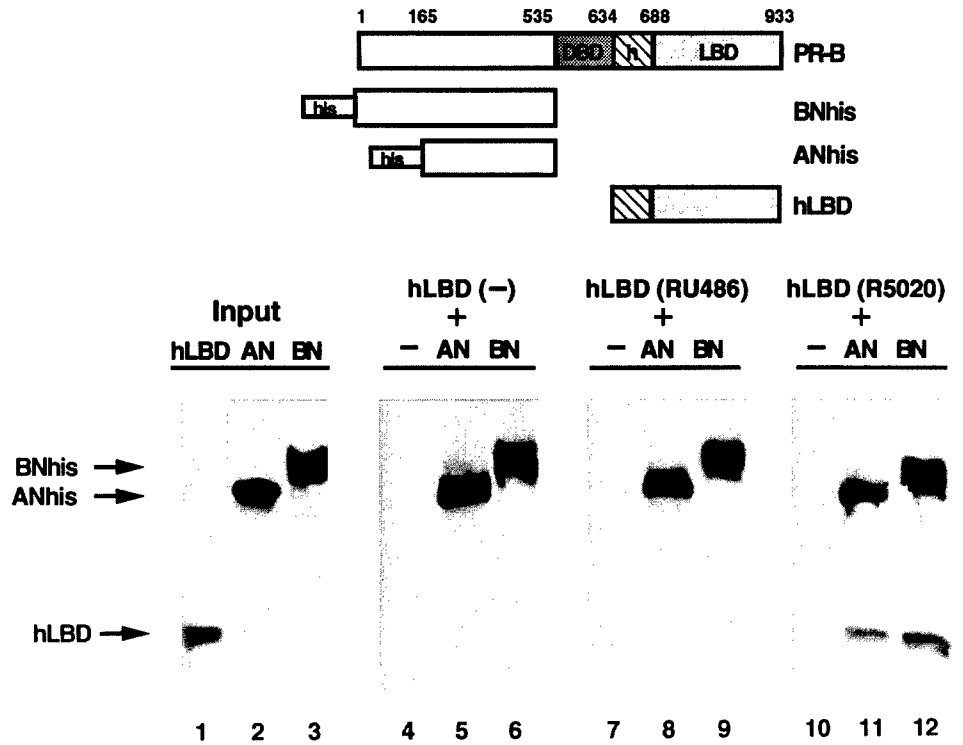

Fig. 2. Interactions of Amino-Carboxyl Domains *in Vitro* are Hormone Agonist Dependent

Whole-cell extracts of Sf9 cells containing the PR domains shown in the schematic were mixed, and association between the N-domains and the hLBD was detected by polyhistidine-tagged pull-down assay as described in Fig. 1. The hLBD was either unliganded (lanes 4–6) or was bound to RU486 (lanes 7–8) or R5020 (lanes 10–12). Proteins bound to Talon resins were eluted and analyzed by Western blot with a mixture of MAbs (AB-52 and C-262) that together detect the N-domains and the hLBD. Inputs (10% of total) of the hLBD and the polyhistidine-tagged N-domain (ANhis and BNhis) are shown in lanes 1–3.

GST pull-down assays similar to that in Fig. 4 revealed that, on average, 5.7% (SEM ± 1.12%, n = 9) of the assay input of the N-domain of PR-B and 6.51% (SEM ± 0.801, n = 9) of the input of the N-domain of PR-A specifically associated with immobilized hLBD-GST. Thus, the more efficient interaction of the hLBD with the N-domain of PR-B, as compared with the N-domain of PR-A that was detected with PR domain polypeptides prepared as crude cell extracts, was not detected by GST pull-down assay with highly purified PR fragments. Whether these different results are due to the use of different assay methods (GST *vs.* polyhistidine pull-down assays) or to the presence of other bridging proteins that facilitate interaction between the N-domain of PR-B and the hLBD is not known. To investigate this question further we analyzed the influence of SRC-1 and CBP on the interaction between purified N- and C-domain PR fragments. SRC-1 and CBP were each expressed as full-length proteins with polyhistidine tags in the baculovirus system and were purified by nickel chelation affinity chromatography. As a control for the general effect of other proteins on the stability of highly purified PR fragments, ovalbumin (10 μg) was added and was observed to have no effect on these *in vitro* interactions (not shown). Addition of SRC-1, CBP, or both proteins together also had no influence on the interactions detected by GST pull-down assay between purified N-domains of PR-A or PR-B with the hLBD (not shown). Thus, we conclude that the N-domains of PR-A and PR-B can make direct protein contact with the hLBD in a manner that does not require SRC-1 or CBP. These results with purified PR fragments also suggest that the more efficient interaction of the hLBD with the N-domain of PR-B, as compared with the N-domain of PR-A observed in whole-cell extracts, is likely due to proteins other than SRC-1/CBP, or to a coactivator complex consisting of SRC-1 and CBP plus additional factors.

Functional Hormone-Agonist Dependent Interaction between the Amino- and Carboxyl-Terminal Domains of PR by Mammalian Two-Hybrid Assay

A mammalian two-hybrid assay was used to determine whether the N-terminal regions of PR-A and PR-B can functionally interact with the C-terminal hLBD within cells. The hybrid protein constructs depicted in Fig. 5 included the hLBD fused to the DBD of Gal4 (hLBD-Gal4) and the N-domains of PR-A (AN-VP16) and PR-B (BN-VP16) fused to the VP16 transcriptional activation domain. An SV40

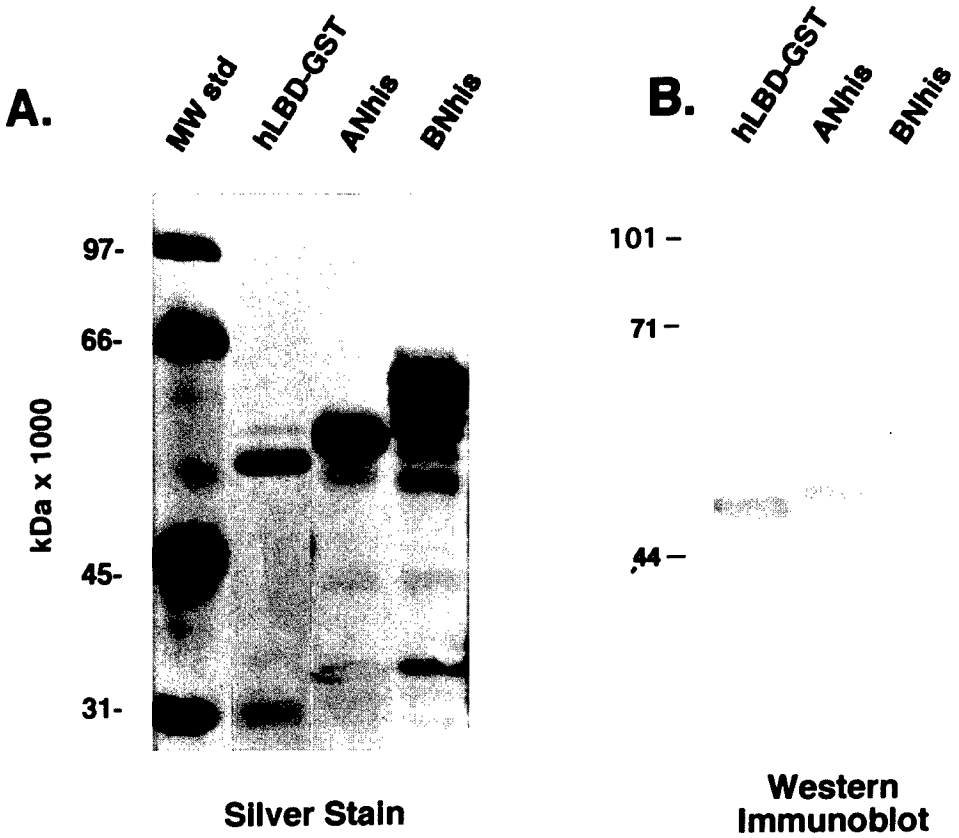

Fig. 3. Purification of PR Domain Polypeptides

Recombinant PR hLBD-GST purified by glutathione Sepharose 4B affinity chromatography, and the N-domains of PR-A (ANhis) and PR-B (BNhis) purified by Ni-NTA affinity chromatography, were analyzed by SDS-PAGE and silver staining (panel A) and by Western blot (panel B) with a mixture of MAbs that recognize epitopes in the N-domain common to PR-A and PR-B (AB-52) and in the LBD (C262).

large T antigen fused to VP16 (T-VP16) was used as a control for nonspecific interaction of the hLBD with an unrelated protein. A luciferase gene inserted downstream of five Gal4 DNA-binding sites (5× Gal4-RE-LUC) was used as the reporter for detection of functional interaction between hLBD-Gal4 and the VP16 fusion proteins (Fig. 5). Human hepatoma (HepG2) or human cervical carcinoma (HeLa) cells were cotransfected with hLBD-Gal4 and one of the three VP16-fusion constructs, and the cells were treated without and with PR ligands for 48 h before harvest and measurement of luciferase activity. Western blot analysis confirmed that the fusion products were expressed as correctly sized proteins and at levels similar to full-length transfected wild-type PR (data not shown). For the experiments in Fig. 6, the nonspecific luciferase expression resulting from interaction between SV40-VP16 and hLBD-Gal4 for each ligand treatment group was normalized to a value of 1.0, and the specific luciferase expression, dependent on both hLBD-Gal4 and PR N-domain VP16 fusion constructs, was calculated as the fold induction over the nonspecific expression of luciferase.

In the absence of ligand, hLBD-Gal4 did not functionally interact in either HepG2 cells (Fig. 6A) or HeLa cells (Fig. 6B) with the N-domain-VP16 fusions of either PR-A (AN-VP16) or PR-B (BN-VP16) above that of the background interaction with SV40-VP16 (T-VP16). However, progesterone addition to both cell types induced a significant functional interaction between hLBD-Gal4 and either PR N-domain VP16 construct (Fig. 6). Additionally, hLBD-Gal4 interacted more efficiently with the N terminus of PR-B (5.2 ± 0.1 fold induction in HepG2 and 6.3 ± 0.7 in HeLa cells) than the N-domain of PR-A (2.7 ± .01 in HepG2 and 4.0 ± 0.3 in HeLa cells; $P < 0.05$) in both cell lines (Fig. 6). In agreement with the *in vitro* protein-protein interaction results, RU486 failed to induce a functional interaction between hLBD-Gal4 and either PR isoform N-domain construct in HepG2 cells (Fig. 6A) and with the N-domain of PR-A in HeLa cells (Fig. 6B). However, in HeLa cells a small but significant RU486 stimulation (1.9 ± 0.3-fold over the T-VP16 control, $P < .01$) of hLBD-Gal4 interaction with the N-domain of PR-B was observed, which was considerably less than that stimulated by progesterone (Fig. 6B). Thus, functional

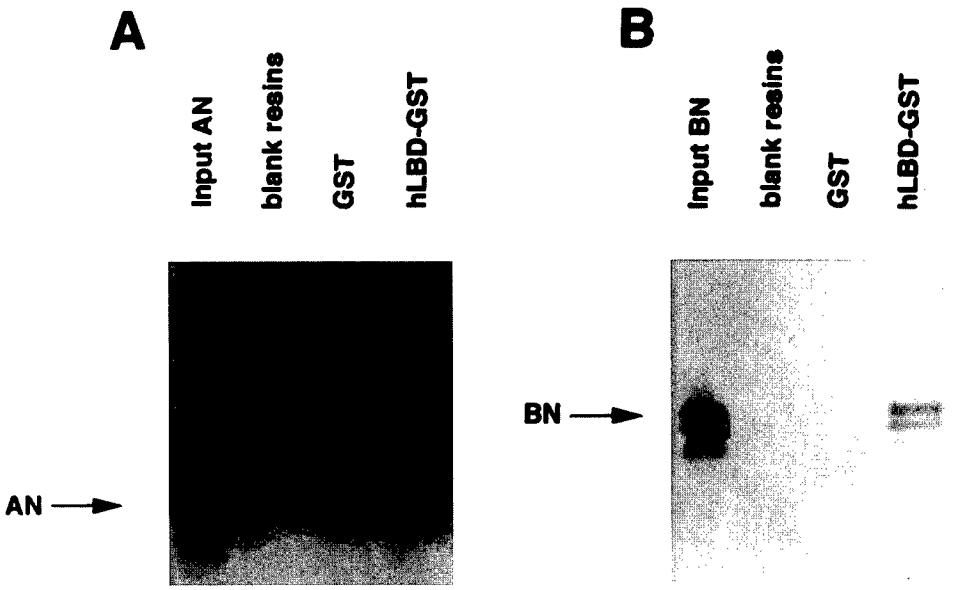

Fig. 4. Direct Interaction *in Vitro* between purified N- and C-Domains of PR

Purified hLBD-GST, or GST as a control, were preimmobilized to glutathione Sepharose. Equal amounts (determined by Western blot) of purified N-domains of PR-A (panel A) or PR-B (panel B) were incubated with the hLBD-GST, GST, or blank resins for 1 h at 4 C. After washing of resins, bound proteins were eluted and analyzed by Western blot with an MAb (1294) that detects an epitope in the N-domain common to PR-A and PR-B. Assay input (10%) of the N terminus of PR-A (AN) or PR-B (BN) for each GST-pull down assay is indicated.

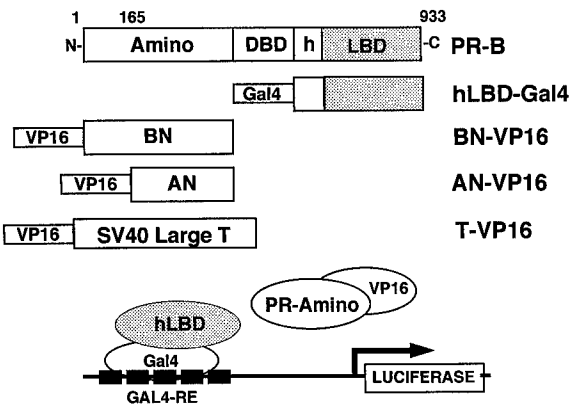

Fig. 5. Mammalian Two-Hybrid Assay Constructs

Schematic diagram of the fusion constructs and reporter gene used in the mammalian two-hybrid assay. The VP16 acidic activation domain (aa 411–455) was fused to the N terminus of PR-B (aa 1–550, BN-VP16), the N terminus of PR-A (aa 165–550, AN-VP16), or the SV40 large T antigen (T-VP16). The Gal4 DBD (aa 1–147) was fused to the PR LBD plus hinge sequences (aa 634–933) to yield hLBD-Gal4. Interaction between the hLBD-Gal4 and N-domain VP16 fusion proteins was measured as an induction of expression of the luciferase reporter gene under the regulation of five Gal4 DNA-binding sites (5× Gal4-RE-LUC).

interaction between the C- and N-domains of PR within mammalian cells is hormone agonist-dependent and is either not allowed or greatly reduced (hLBD interaction with the N-domain of PR-B in HeLa cells) by the antagonist RU486.

Coactivators Are Involved in Functional Interaction between Amino- and Carboxyl-Terminal Domains of PR within Whole Cells

To investigate the role of transcriptional coactivators in the functional interaction between the N- and C-domains of PR, we analyzed whether coexpression of full-length SRC-1, CBP, or both proteins would influence these interdomain interactions in the mammalian two-hybrid assay. Separate cotransfections with either SRC-1 or CBP in HepG2 (Fig. 7) or HeLa cells (data not shown) had minimal effect on progesterone-dependent interaction between hLDB-Gal4 and the N-domain VP16 constructs of PR-A and PR-B. However, cotransfection with SRC-1 and CBP together resulted in a significant stimulation of progesterone-dependent functional interaction between hLBD-Gal4 and the N-domains of PR-A or PR-B (Fig. 7). In HepG2 cells, cotransfected SRC-1 and CBP together increased hLBD-Gal4 interaction with PR-B N-domain from a 4.6- to a 13-fold induction (2.8×) and hLBD-Gal4 interaction with PR-A N-domain from a 3.2 to a 6.6 fold induction (2.06×) (Fig. 7). A similar enhancement of functional interaction between hLBD and the N terminus of PR-B (3.37-fold increase) and the N-terminus of PR-A (4.41-fold increase) was observed by cotransfecting HeLa cells with SRC-1 and CBP (not shown). Enhancement by SRC-1 and CBP is largely PR specific and does not appear to be due to a coactivation effect on general transcription. Coexpression of SRC-1 and CBP together resulted in only a 1.4-

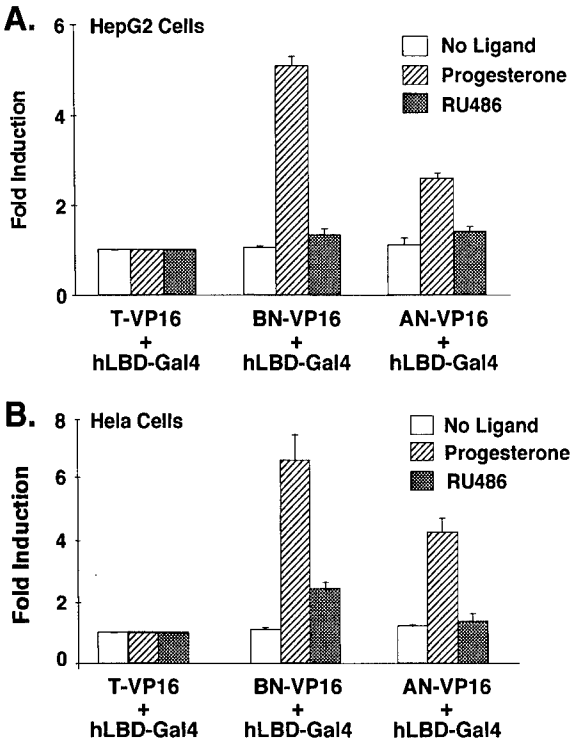

Fig. 6. Hormone Agonist-Dependent Functional Interaction between Amino and Carboxyl Domains of PR

HepG2 cells (A) or HeLa cells (B) were transiently cotransfected with hLBD-Gal4 and VP16 fusion constructs containing the N-domains of PR-B (BN-VP16), PR-A (AN-VP16), or the SV40 large T antigen (T-VP16). Transcriptional activity of the luciferase gene was assayed on the 5× Gal4-RE-LUC reporter as a measure of functional interaction between Gal4 and VP16 fusion proteins. Transcriptional activity was measured in the absence of ligand or in the presence of progesterone (10^{-7} M) or the progesterone antagonist RU486 (10^{-7} M). The data are represented as fold induction over the control interaction between hLBD-Gal4 and T-VP16 for each ligand treatment group that was normalized to 1.0. *Bars* are the mean ± SEM from three independent experiments.

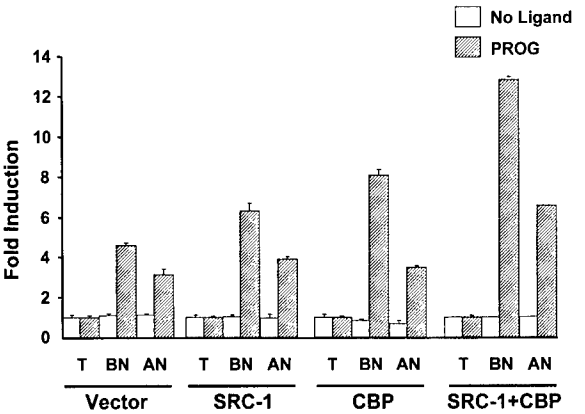

Fig. 7. Coexpression of Coactivators Enhances Functional Interaction between Amino and Carboxyl Domains of PR

HepG2 cells were transiently transfected with hLBD-Gal4 and the VP16 fusion constructs expressing SV40 large T antigen (T), or the N-domains of PR-B (BN) or PR-A (AN), as in Fig. 6, except in the absence (empty pCR3.1 vector) or presence of expression vectors for SRC-1, CBP, or both coactivators. The data are calculated as fold inductions over the control interaction between hLBD-Gal4 and T-VP16 for each group and are the mean of triplicate determinations (±SEM) from a single representative experiment.

to 1.5-fold stimulation of Gal4-VP16 transactivation of the Gal4-RE-LUC reporter gene in both HeLa and HepG2 cells, indicating that SRC-1 and CBP are not affecting transcription activation in general (not shown).

To test the extent to which coactivators are essential for functional interaction between N- and C-domains of PR, the activities of endogenous SRC-1 and CBP were inhibited in the mammalian two-hybrid assay. To inhibit SRC-1, cells were cotransfected with a dominant-negative form of SRC-1 (0.8) that contains the C-terminal nuclear receptor-binding site enabling it to bind to PR, but lacks the centrally located nuclear receptor-binding sites and both transcriptional activation domains (16, 28). Coexpression of SRC-1 (0.8) in the mammalian two-hybrid assay effectively inhibited progesterone-dependent interaction between hLBD-Gal4 and the N-domains of either PR isoform (Fig. 8A). To inactivate endogenous CBP, cells were cotransfected with an expression plasmid for the adenovirus protein 12S E1A (E1A), which binds to the third zinc finger motif of CBP and inactivates its coactivator function (49). E1A cotransfection in the mammalian two-hybrid assay effectively inhibited progesterone induction of the functional interaction between hLDB-Gal4 and the N-domains of either PR isoform (Fig. 8B). As a control for effects on general transcription activation, the dominant negative SRC-1 (0.8) and E1A did not affect the constitutive transactivation of the GAL4-RE-LUC reporter mediated by a Gal4-VP16 activator (not shown). These mammalian two-hybrid results, taken together with the *in vitro* protein-protein interaction data, suggest that SRC-1 and CBP are essential for functional hormone-dependent interaction between the amino- and carboxyl-terminal domains of PR, but are not required as bridging, or adaptor, proteins for association between the N- and C-domains, which occurs by direct contact in the absence of other proteins.

DISCUSSION

Full transcriptional activity of steroid receptors requires functional synergy between activation functions located in the amino and carboxyl domains of receptor (4, 5, 32–39). Previous studies with ER (40) and AR (30, 41–44) using a standard or modified two-hybrid assay, suggest that this functional synergy involves a ligand-dependent association between the amino and carboxyl domains of receptor. Using a mammalian two-hybrid interaction system, we have also observed a

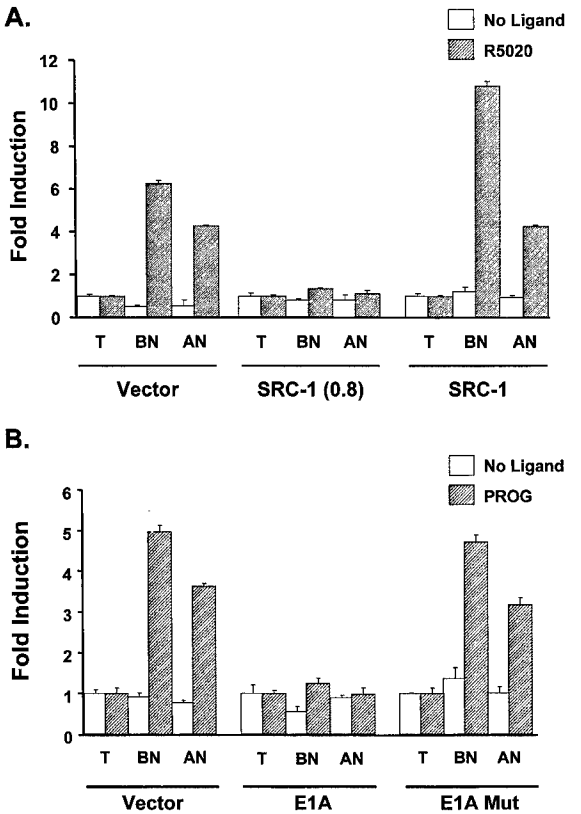

Fig. 8. Inactivation of Endogenous Coactivators Inhibits Functional Two-Hybrid Interaction Between N- and C-Domains of PR

HeLa cells were cotransfected with hLBD-Gal4 and VP16 fusion constructs (T-VP16, AN-VP16, and BN VD16) as in Figs. 6 and 7, except that cells were also transfected with and without (empty vector) a dominant negative SRC-1 (0.8) (panel A) or the adenovirus protein E1A (panel B). Luciferase activity was normalized to β-galactosidase activity and calculated as in Figs. 6 and 7. The data are mean values of triplicate determinations (± SEM) from a single representative experiment.

hormone-agonist dependent functional interaction between N-terminal domains and the hLBD of human PR, suggesting that N-C interdomain interaction is a common mechanism for all steroid hormone receptors. An unresolved question from previous two-hybrid results is whether the observed functional interaction represents direct protein contacts or is indirectly mediated by other proteins that associate with either N- or C-domains of the receptor. To resolve this question we have investigated the ability of N- and C-domain polypeptides of PR to interact directly *in vitro*. When expressed as recombinant polypeptides in Sf9 cells and prepared as whole-cell extracts, the N-domains of both PR isoforms interacted efficiently with the C-terminal hLBD of PR. Furthermore, this *in vitro* interaction was dependent on the hormone agonist R5020 and was not detected in the absence of ligand (Figs. 1 and 2). When the N- and C-domain polypeptides were purified to more than 90% from Sf9 whole-cell extracts, they continued to interact by pull-down assay in a specific manner indicating that the N- and C-domains of PR are capable of making direct protein contacts and do not require other proteins to physically associate.

While the interaction between the amino- and carboxyl domains of PR *in vitro* (Fig. 2 and Table 1), and within cells by mammalian two-hybrid assay, was observed to be hormone agonist dependent (Fig. 6), little or no interaction was detected by either experimental approach in the presence of the progesterone antagonist RU486. Androgen antagonists were similarly reported to diminish functional interaction between the N- and C-domains of AR in a mammalian two-hybrid assay (42). However, different results were observed for the effects of the antiestrogen *trans*-hydroxytamoxifen (TOT), on ER N-C domain interactions. Separately expressed ER polypeptides containing the amino terminus linked to the DBD and the LBD were observed to functionally interact on an estrogen response element (ERE)-controlled reporter gene in response to estradiol, but not to TOT. In contrast, TOT was observed to induce a strong functional interaction between the N-terminal DBD construct and the LBD on the ERE-responsive reporter gene when the LBD was fused to VP16 (40). Because TOT only induced a response between the N- and C-domains when the LBD was expressed as a fusion protein with VP16, it has been suggested that TOT produces a nonproductive interaction between the N- and C-domains (40). A different conclusion must be drawn from the present studies for the influence of RU486 on PR N-C domain interactions, since RU486 failed to induce an interaction between the N-domains and the hLBD of PR *in vitro* (Fig. 2) and functionally inhibited hLBD interaction with N-domain VP16 fusion construct in whole cells by mammalian two-hybrid assay (Fig. 6). Thus, we conclude that RU486 fails to induce, or impairs, a physical association between the N- and C-domains of PR, rather than promoting an interaction that is transcriptionally nonproductive as reported for the effect of TOT on ER N-C domain interaction (40). The reason for the apparent difference between RU486 and TOT is not known. This could be due to differences in assay methods, or to RU486 antagonism of PR operating by a different mechanism than TOT antagonism of ER. Indeed, TOT is well known to exhibit partial agonist effects that are both cell type and promoter dependent, suggesting this difference between TOT and RU486 may reflect the partial agonist effects of TOT. In this regard, RU486 exhibits cell- and promoter-specific partial agonist effects that are mediated solely by the B isoform of PR (4, 7, 10). RU486 stimulated a weak functional interaction between the N terminus of PR-B and the hLBD in HeLa cells that was not observed in HepG2 cells (Fig. 6). This weak RU486 stimulation of N-C interaction correlates with the previously reported weak agonist activity of RU486 mediated by full-length PR-B in HeLa cells on selected promoters (4, 9). Many studies have revealed that ago-

nists and antagonists induce distinct conformational changes in the LBD of steroid receptors and that these conformations are central to whether receptor is transcriptionally active or inactive (50–53). Therefore, an altered conformation in the LBD of PR induced by RU486 may contribute to inactivation of receptor by not permitting an efficient physical association between the amino and carboxyl domains.

The p160 family of nuclear receptor coactivators was initially identified as AF-2-interacting proteins and has been shown to interact with AF-2 as a complex of coactivators consisting minimally of p160 as the direct binding component, CBP, and pCAF (CBP-associated factor) (17–22). The p160 proteins, SRC-1 and GRIP1, have also been found to be capable of interacting with and mediating coactivation effects through N-terminal regions of ER and PR (28–31). Interestingly, separate regions of p160 proteins interact with N- and C-domains of receptors, suggesting that p160 proteins are capable of mediating, or bridging, an association between the N- and C-domains of the receptor (Ref. 31 and V. Boonyaratanakornkit and D. P. Edwards, unpublished). To address the role of coactivators in terms of N-C-domain interactions of PR, the present study analyzed the influence of SRC-1 and CBP on direct N-C domain binding *in vitro* with purified PR fragments and functionally by mammalian two-hybrid assay. Addition of SRC-1, CBP, or both proteins together had no effect on the direct interactions between purified N- and C-domains of PR. However, when cells were cotransfected with SRC-1 and CBP expression plasmids together, functional hormone-dependent interaction between the N- and C-domains of PR in the mammalian two-hybrid assay was enhanced (Fig. 7). Additionally, inactivation of endogenous SRC-1 by transfecting cells with a dominant negative mutant form of SRC-1 (16), or inactivation of CBP with EIA (49), effectively inhibited functional interaction between the N- and C-domains (Fig. 8). The influence of the dominant negative SRC-1 does not preclude other closely related nuclear receptor coactivators from having a role in mediating a functional N-C domain interaction. The dominant negative SRC-1 may compete with other coactivators containing the same nuclear receptor interaction box sequences (LXXLL motif) that bind AF-2 in the LBD. These direct *in vitro* binding and functional two-hybrid results, taken together, are consistent with the conclusion that the N- and C-domains of PR are capable of making direct protein contact without the aid of coactivators, but that transcriptionally productive interactions require both SRC-1 (or closely related coactivators) and CBP.

Although SRC-1, CBP, or both proteins had no influence on interactions between purified N- and C-domain PR fragments, we observed that CBP addition to the PR domain polypeptides in crude extracts of Sf9 cells increased N-C domain interactions (not shown). Since coactivators appear to exist as preformed multiprotein complexes containing SRC-1, CBP, pCAF, and other factors (17), this result suggests the possibility that CBP, as a component of a larger protein complex, can facilitate or stabilize direct associations between the C and N terminii of PR.

When comparing the interaction of the hLBD with the N-domains of the two forms of PR, the N-domain of PR-B was found to interact more efficiently than the N-domain of PR-A. This differential interaction was detected functionally by mammalian two-hybrid assay and *in vitro* by pull-down assays with PR domain polypeptides prepared as whole-cell extracts of Sf9 cells. However, this differential was not observed *in vitro* with highly purified PR domain polypeptides, suggesting that the more efficient interaction of the N-domain of PR-B with the hLBD is dependent on other proteins, most likely coactivator complexes containing SRC-1, CBP, and other components. Additionally, the more efficient interaction observed between the hLBD and the N terminus of PR-B, as compared with the N terminus of PR-A, could be due to 1) additional protein contact sites provided by the extended N-terminal segment unique to PR-B; 2) a different overall conformation conferred by the unique N terminus of PR-B on sites that are common to the N-domains of PR-A and PR-B; or 3) the three phosphorylation sites that are located in the N-terminal segment unique to PR-B (48). Further studies are required to distinguish between these possibilities. The more efficient interaction of the hLBD with the N terminus of PR-B, compared with the N-terminus of PR-A, under the conditions observed in this study, correlates with PR-B functioning as a generally stronger transcriptional activator than PR-A (7–12). These results support the notion that a differential association between the C-terminal hLBD and the N terminus of PR-A and PR-B contributes to the experimentally observed differences in transcriptional activities of the two PR isoforms.

Because the N- and C-domains of PR were expressed as separate polypeptides, the present results cannot distinguish between an intramolecular association between the N and C termini in the full-length receptor and an intermolecular interaction resulting from antiparallel dimerization as suggested by studies with AR (41, 54). Several lines of evidence indicate that PR homodimerization occurs in a parallel fashion, thus supporting the notion that the observed N-C domain interactions reflect an intramolecular association. For example, we and others have shown that the C-terminal hLBD of PR is capable of mediating homodimerization in the absence of N-terminal sequences (45, 55). Furthermore, fusion of the leucine zipper of c-*fos* or c-*jun* to the C terminus of full-length PR forced parallel dimers that were transcriptionally active (56). However, whether *fos/jun*-forced antiparallel dimers are also active was not tested. Additionally, the recently published three-dimensional structure of the

LBD of PR bound to agonist revealed the presence of a dimer interface that mediates parallel interactions through the C terminus (57). As a further suggestion that interactions between isolated N- and C-domains detected in this study *in vitro* and *in vivo* by mammalian two-hybrid assay reflect an intramolecular interaction within the holoreceptor, the N- and C-domains of PR coexpressed in mammalian cells attached to their own DBD were observed to reconstitute a functional transcriptional response *in trans* on a progesterone response element-containing reporter gene (28). Furthermore, cotransfection with SRC-1, or the closely related TIF-2, markedly enhanced this transcriptional response.

The hLBD was capable of interacting with the N-domain of PR *in vitro*, while the LBD was not (Fig. 1), suggesting the hinge region is involved in N-C domain interactions. Whether hinge sequences are directly involved in protein interaction with N-domain fragments has not been investigated. Although a direct involvement remains a possibility, we favor the idea that the hinge exerts an effect on the conformation of the LBD enabling it to make protein contacts with N-domains. Although studies to show directly whether the hinge confers structural stability on the PR LBD have not been performed, indirect functional studies comparing the LBD and hLBD fragments are consistent with this role for the hinge. We have shown previously that the expressed LBD alone is not capable of mediating homodimerization and binds ligand with an affinity that is 3- to 4-fold lower than the affinity of full-length receptor. The LBD with additional hinge sequences is the minimum region of PR capable of binding ligand with wild-type affinity and mediating homodimerization (45).

In Fig. 9 we have modeled our findings in the context of full-length PR. We propose that a fully active receptor requires assembly of AF-1 and AF-2 from different regions of the same PR polypeptide. Receptor bound to agonist undergoes a conformational change that allows a direct intramolecular association between the N- and C-domains (*dashed lines*). The p160 subunit of the transcriptional coactivator complex is capable of simultaneously binding with amino (AF-1) and carboxyl (AF-2) regions of receptor, and this complex is required for a transcriptionally productive interaction between the N- and C-domains. The N terminus of PR-B interacts more efficiently with the hLBD than the N terminus of PR-A, suggesting that differential N-C domain interactions contribute to the distinct functional activities of PR-A and PR-B. This differential interaction appears to be facilitated by protein components (*checkered symbol*) of a coactivator complex through the extended N-terminal segment of PR-B. Direct N-C domain interactions are markedly inhibited in the presence of RU486, suggesting that failure to induce an association between the N- and C-domains contributes to the mechanism by which antagonists inactivate the receptor.

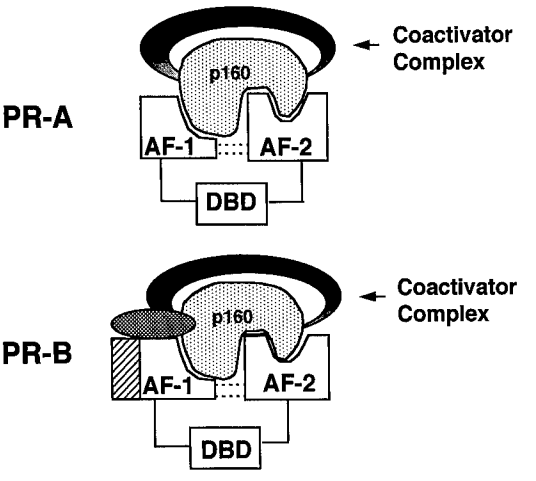

Fig. 9. Model of Hormone Agonist-Dependent Intramolecular Association of Amino and Carboxyl Domains of PR

The three major domains of PR-A and PR-B are indicated schematically: the amino-terminal domain containing AF-1, the DBD, and the carboxyl-terminal LBD containing AF-2. The model depicts the hormone agonist-activated PR with the *stippled* region representing the N-terminal extended segment unique to PR-B. The *dashed lines* represent direct contacts between N- and C-domains, and the coactivator complexes associated with PR-A and PR-B contain distinct subunit compositions.

MATERIALS AND METHODS

Materials

Unlabeled progesterone and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). Unlabeled RU486 (Mifepristone, 17-hydroxy-11 [4-dimethlyaminophenyl] 17-propynyl-estra-4, 5-diene-3-one) was a gift from Roussel-UCLAF (Romainville, France) or Ligand Pharmaceuticals, Inc. (San Diego, CA). Nickel-NTA (Ni-NTA) and metal ion affinity resins (Talon) were obtained from Qiagen (Valencia, CA) and CLONTECH Laboratories, Inc. (Palo Alto, CA), respectively. Mouse IgG1 MAbs generated against human PR include AB-52 and 1294, which recognize epitopes in the amino terminus common to PR-A and PR-B (Ref. 46 and B. Spaulding, L. Sherman, and D. P. Edwards, unpublished data), B-30, which recognizes only PR-B (46), and C-262, which is directed against the last 14 amino acids of the carboxyl-terminal end of PR (47). A polyclonal antibody raised against PR-A (B13-TK) was a gift from Nancy Weigel (Baylor College of Medicine, Houston, TX). A MAb generated against the polyhistidine tag and enterokinase cleavage site fusion sequences (mouse IgG clone 1162/F6) contained in the pBlueBacHis2 baculovirus transfer plasmid (Invitrogen) was used for Western blot detection of baculovirus polyhistidine-tagged proteins and a MAb produced to GST (mouse IgG clone 794/H12) was used for Western blot detection of GST-fusion proteins (D. P. Edwards and S. Anderson, unpublished data). Secondary antibodies, Hybond-C Extra (nitrocellulose) transfer membrane, and x-ray developing film were obtained from Amersham (Arlington Heights, IL). DNA restriction and modification enzymes were obtained from Promega Corp. (Madison, WI), Boehringer Mannheim (Indianapolis, IN), or New England Biolabs, Inc. (Beverly, MA). PCR reagents were obtained from Perkin-Elmer Corp. (Norwalk, CT) or Promega Corp.

Expression of PR Fragments and Coactivators in the Baculovirus Insect Cell System

Recombinant baculovirus vectors expressing different domains of PR with N-terminal polyhistidine tags (6×) (Fig. 1) included the N terminus of PR-B (BNhis, aa 1–535), the N-terminus of PR-A (ANhis, aa 165–535), and the hLBD (aa 634–933). The LBD alone (aa 688–933) was expressed without polyhistidine tags. These vectors have been described and used previously (45) except for BNhis, which was constructed by restriction digestion of PR-B from plasmid pH PR-B (7, 59) by *Eco*NI, which dropped out the base pair 1779–2671 fragment of PR-B cDNA. The *Eco*NI ends were made blunt by digestion with Mung Bean nuclease and then religated resulting in a cDNA encoding a PR fragment, aa 1–535. For expression of the hLBD as a fusion protein containing an amino-terminal GST tag (hLBD-GST), the hLBD was generated by PCR with the primers 5′-GATCGGATCCGGCATGGTCCTTG GAGGT and 5′-CTAGAATCCAAAGATGACATTCACTTTTTATG, using the pT7BhPR-A plasmid (provided by M. Tsai and B. O'Malley, Baylor College of Medicine, Houston, TX) as the template cDNA (50). The PCR amplification product resulted in aa 634–933 of PR containing *Bam*HI and *Eco*RI restriction sites at the 5′- and 3′-ends, respectively, which was ligated into the respective restriction sites of the pAcG2T baculovirus transfer vector (PharMingen, San Diego, CA).

A recombinant baculovirus transfer vector for steroid receptor coactivator-1 (SRC-1) (16) was constructed by inserting the SRC-1 cDNA excised from pBK-CMVSRC-1 (provided by Sergio Oñate, M.-J. Tsai and B. O'Malley, Baylor College of Medicine) into *Bam*HI and *Pst*I sites of the baculovirus transfer plasmid pBlueBacHis2(C) (Invitrogen). The SRC-1 coding region was inserted in frame with amino-terminal sequences of the plasmid containing an ATG translation start site, six sequential histidine residues, and an enterokinase cleavage site encoding aa 361-1440 of SRC-1 (SRC-1 his). The recombinant virus for expression of full-length mouse CBP as an N-terminally polyhistidine-tagged protein (CBPhis) was provided by N. Weigel and B. O'Malley (Baylor College of Medicine).

Spodoptera frugiperda (Sf9) insect cells were grown in spinner vessels (150–500 ml) in Graces' insect cell medium supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT). Cells were infected with recombinant viruses at a multiplicity of infection of 1.0 for 48 h at 27 C as described previously (51, 58). Insect cell cultures for expression of C-terminal PR fragments were incubated with 200 nM R5020 or RU486, as indicated, for the final 6 h of infection before harvest.

Purification of Baculovirus-Expressed PR Domains and Coactivators

The N-terminal domains of PR-A and PR-B expressed in baculovirus with a polyhistidine tag (ANhis and BNhis) were purified by metal ion affinity chromatography as described previously (58, 59) with minor modifications. Sf9 cells expressing either ANhis or BNhis were lysed in 20 mM Tris and 10% glycerol (TG) buffer, pH 8.0, containing 350 mM NaCl, 15 mM imidazole, 1 mM β-mercaptoethanol, and a mixture of protease inhibitors (59). All procedures were done at 0–4 C. Cell lysates were centrifuged at 100,000 × *g* for 30 min, and the supernatant was taken as a soluble whole-cell extract. Whole-cell extracts were bound to nickel affinity resins (1 ml packed Ni-NTA resins) by resuspension in a 50-ml siliconized tube followed by incubation for 1 h on an end-over-end rotator. The resins were then washed four times by centrifugation (1500 rpm) with lysis buffer. The resins were washed once more in lysis buffer lacking salt and then transferred to a 2-ml siliconized tube. Bound proteins were eluted from the resin by suspension in lysis buffer containing 100 mM imidazole, and the supernatant containing the eluted protein was collected by centrifugation. Eluates were stored at −80 C in aliquots and analyzed by Lowry assay for protein concentration, by silver-stained SDS-PAGE for purity, and by Western blot for identification of purified products. CBPhis and SRC-1 his were purified using the same procedure except that the lysis buffer contained 2 mM imidazole.

The hLBD-GST fusion protein was purified by glutathione Sepharose affinity chromatography. Whole-cell extracts were made in cell lysis buffer (10 mM Tris-base, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, containing 350 mM NaCl) as described above and bound to glutathione Sepharose 4B resins (Pharmacia Biotech, Piscataway, NJ) by resuspension in a 50-ml siliconized tube for 2 h on an end-over-end rotator. The resins were washed four times by centrifugation (1500 rpm) with lysis buffer. The resins were washed once more in lysis buffer lacking salt, and then transferred to a 2-ml siliconized tube. Bound proteins were eluted with 20 mM glutathione and collected by centrifugation. Eluted samples were analyzed as described above.

Pull-Down Assays to Detect PR Domain Interactions *in Vitro*

For experiments in crude extracts, Sf9 cells expressing different PR domains were lysed as above, and whole cell extracts were dialyzed against lysis buffer lacking salt. PR hLBDhis was treated with EnterokinaseMax (Invitrogen) to cleave off the N-terminal polyhistidine tag as described previously (45). Sf9 whole-cell extracts were added to the hLBD lacking the his-tag, which was then dialyzed against lysis buffer without salt. The hLBD was analyzed by Western blot with the PR-specific MAb C-262 and the anti-his tag MAb (1162/F6) to confirm removal of the his-tag (data not shown). The PR LBD was expressed as a non-his-tagged protein and prepared as whole-cell extracts for Sf9 cells. The LBD or hLBD was incubated with polyhistidine-tagged N-terminal domain polypeptides of PR-A (ANhis), PR-B (BNhis), or buffer, which served as a control for nonspecific binding of non-his LBD or hLBD to metal resins, in siliconized microcentrifuge tubes for 30 min on ice. TG buffer (20 mM Tris-HCl, pH 8.0, plus 10% glycerol) containing 45 mM imidazole and 300 mM NaCl was added to bring the final imidazole concentration to 15 mM imidazole and NaCl to 100 mM. One hundred microliters of a 1:1 suspension of Talon (CLONTECH Laboratories, Inc.) metal affinity resin or Ni-NTA resin (Qiagen) were added to each tube. Samples were then resuspended and incubated in batch at 4 C for 1 h on an end-over-end rotator followed by washing of the resins four times by centrifugation in TG buffer containing 15 mM imidazole and 100 mM NaCl. Resins were transferred to a new microcentrifuge tube and washed twice more. Bound proteins were extracted with 2% SDS sample buffer and electrophoresed on 10% or 7.5% polyacrylamide SDS gels as previously described (45–47). Separated proteins were transferred to nitrocellulose paper and detected by Western blot assays with a mixture of MAbs including C-262 generated against the C terminus and AB-52 generated against the N terminus common to PR-A and PR-B (46, 47). [^{35}S]protein A (Amersham) and autoradiography were used as the detection methods as described previously (45).

For experiments using purified receptors, a GST pull-down assay was developed that was similar to the polyhistidine pull-down assay except for the following modifications. The purified hLBD-GST was bound to 100 μl of a 1:1 suspension of glutathione Sepharose 4B resin, which had been pretreated with ovalbumin (5 μg/100 μl of resin) for 15 min, on an end-over-end rotator for 1 h at 4 C in TG buffer containing 100 mM NaCl. The resins were washed once by centrifugation with TG buffer containing 100 mM NaCl. Ten micrograms of ovalbumin and either purified ANhis or BNhis were added to the sample. TG buffer containing 300 mM NaCl was added to bring the final concentration of NaCl to 100 mM. Samples

were incubated on an end-over-end rotator for 1 h at 4 C and then washed by centrifugation once with TG containing 100 mM NaCl, twice with TG containing 125 mM NaCl, and once more with TG containing 100 mM NaCl. Resins were transferred to a new microcentrifuge tube and washed twice more with TG containing 100 mM NaCl. Bound proteins were eluted and analyzed as described above for polyhistidine pull-down assay.

Mammalian Two-Hybrid Assay

The PR hLBD (aa 634–933) was cloned as a fusion protein at the amino terminus with Gal4-DBD (aa 1–147) into the pBK-CMV mammalian expression vector (Stratagene, La Jolla, CA) as described previously (11). The amino terminus of PR-A (aa 165–550) and PR-B (aa 1–550) were cloned into the pVP16 fusion vector (CLONTECH Laboratories, Inc.) to yield AN-VP16 and BN-VP16, respectively, as follows: the fusion constructs Gal4-DBD-BN and Gal4-DBD-AN were digested with *Eco*RI and *Xba*I, and the coding sequences for the respective PR domains were ligated into pVP16, previously digested with *Eco*RI and *Xba*I. A control vector for nonspecific protein interaction contained the SV40 large T antigen fused to VP16 (T-VP16) and was purchased from CLONTECH Laboratories, Inc. The luciferase reporter gene contained a TATA box and five copies of the Gal4 DNA-binding sites (5× Gal4-TATA-LUC, a gift from X. F. Wang, Duke University, Durham, NC). Mouse CBP cDNA was excised from pRc/RSV-mCBP-HA-RK (a gift from R. Goodman, Oregon Health Sciences Center, Portland, OR) (60) by digestion with *Hin*dIII and *Not*I. The full-length CBP cDNA was then inserted into the *Hin*dIII and *Not*I restriction sites of pCR3.1 mammalian expression vector (Invitrogen) to yield pCR3.1-CBP, which expresses full-length mouse CBP with an HA (hemagglutinin antigen) tag. pCR3.1-SRC-1 and SRC-1(0.8) were gifts from B. W. O'Malley (Baylor College of Medicine). The mammalian expression vector for E1A (pbcl2-E1A12S) was a gift from J. Nevins (Duke University).

HeLa cells and HepG2 cells were maintained in MEM plus 10% FCS (Life Technologies, Gaithersburg, MD). Cells were plated in 24-well dishes (coated with 0.1% gelatin for HepG2 cells) and allowed to grow 24 h before transfection. DNA was introduced into the cells using Lipofectin (Life Technologies). Briefly, triplicate transfections were performed using 3 μg of total DNA. For standard transfections 50 ng of pBKC-β-gal (normalization vector) (61), 500 ng of reporter (5× Gal4-TATA-LUC), 1000 ng of hLBD-Gal4, 1000 ng of VP16 fusion constructs, and 450 ng of pCR3.1, 450 ng pCR3.1-hSRC-1, 450 ng pCR3.1-CBP, or a combination of 225 ng of pCR3.1-CBP and 225 ng of pCR3.1-SRC-1 (total of 450 ng of plasmid) were used. Cells were incubated with Lipofectin for 3 h, at which time media were removed and cells were treated with the appropriate hormone diluted in phenol red-free media containing 10% charcoal-stripped FCS (HyClone Laboratories, Inc., Logan, UT). Incubation with hormone continued for 48 h, after which cells were lysed and assayed for luciferase and β-galactosidase activity as described previously (62).

Data Analysis

Comparisons of results from protein-tagged pull-down and mammalian two-hybrid assays were done by Student's *t* tests or ANOVA using Excel 5.0 (Microsoft Corp.) to determine whether there was a significant difference among groups. Results were considered statistically significant at $P < 0.05$.

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Address requests for reprints to: Dean P. Edwards, Ph.D., Department of Pathology, B216, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, Colorado 80262. E-mail: Dean.Edwards@UCHSC.edu.

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*Present address: Center for Neuroendocrine Studies, Tobin Hall, Box 37720, University of Massachusetts, Amherst, Massachusetts 01003.

†Equal contributors to this work and should both be considered as first authors.

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The Novel Progesterone Receptor Antagonists RTI 3021–012 and RTI 3021–022 Exhibit Complex Glucocorticoid Receptor Antagonist Activities: Implications for the Development of Dissociated Antiprogestins*

B. L. WAGNER†, G. POLLIO, P. GIANGRANDE‡, J. C. WEBSTER, M. BRESLIN, D. E. MAIS, C. E. COOK, W. V. VEDECKIS, J. A. CIDLOWSKI, AND D. P. McDONNELL

Department of Pharmacology and Cancer Biology (B.L.W., G.P., P.G., D.P.M.), Duke University Medical Center, Durham, North Carolina 27710; Molecular Endocrinology Group (J.C.W., J.A.C.), NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709; Department of Biochemistry and Molecular Biology (M.B., W.V.V.), Louisiana State University Medical School, New Orleans, Louisiana 70112; Ligand Pharmaceuticals, Inc. (D.E.M.), San Diego, California 92121; Research Triangle Institute (C.E.C.), Chemistry and Life Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

We have identified two novel compounds (RTI 3021–012 and RTI 3021–022) that demonstrate similar affinities for human progesterone receptor (PR) and display equivalent antiprogestenic activity. As with most antiprogestins, such as RU486, RTI 3021–012, and RTI 3021–022 also bind to the glucocorticoid receptor (GR) with high affinity. Unexpectedly, when compared with RU486, the RTI antagonists manifest significantly less GR antagonist activity. This finding indicates that, with respect to antiglucocorticoid function, receptor binding affinity is not a good predictor of biological activity. We have determined that the lack of a clear correlation between the GR binding affinity of the RTI compounds and their antagonist activity reflects the unique manner in which they modulate GR signaling. Previously, we proposed a two step "active inhibition" model to explain steroid receptor antagonism: 1) competitive inhibition of agonist binding; and 2) competition of the antagonist bound receptor with that activated by agonists for DNA response elements within target gene promoters. Accordingly, we observed that RU486, RTI 3021–012, and RTI 3021–022, when assayed for PR antagonist activity, accomplished both of these steps. Thus, all three compounds are "active antagonists" of PR function. When assayed on GR, however, RU486 alone functioned as an active antagonist. RTI 3021–012 and RTI 3021–022, on the other hand, functioned solely as "competitive antagonists" since they were capable of high affinity GR binding, but the resulting ligand receptor complex was unable to bind DNA. These results have important pharmaceutical implications supporting the use of mechanism based approaches to identify nuclear receptor modulators. Of equal importance, RTI 3021–012 and RTI 3021–022 are two new antiprogestins that may have clinical utility and are likely to be useful as research reagents with which to separate the effects of antiprogestins and antiglucocorticoids in physiological systems. (*Endocrinology* **140:** 1449–1458, 1999)

THE STEROID HORMONE progesterone is a key regulator of the processes involved in the development and maintenance of reproductive function (1). However, the efficacy of antiprogestins as treatments for brain meningiomas, breast cancer, uterine fibroids, and endometriosis have implicated progesterone in the pathology of these diseases (2–9). Consequently, although a relatively new class of molecules, the antiprogestins are likely to have a wide range of clinical applications. The most widely used antiprogestin, RU486 (mifepristone), was originally developed as an antiglucocorticoid but was subsequently shown to be a potent and effective antiprogestin (10). As an antiprogestin, RU486 is used to induce medical abortions and as a missed menses inducer (11, 12). For these applications, the drug is given acutely and, consequently, the antiglucocorticoid activity is unlikely to cause any lasting side effects. For chronic administration, however, such as would be required for most endocrinopathies, it is likely that the antiglucocorticoid activity of these compounds would not be desirable. Therefore, there has been a great deal of interest in developing compounds that will inhibit progesterone receptor (PR) transcriptional activity but do not interfere with the biological actions of glucocorticoids.

All of the currently available antiprogestins are steroidal in nature and are derived from a 19-nor testosterone backbone (10, 13, 14). It is likely that nonsteroidal antiprogestins with improved selectivity will be developed. In their absence, efforts to dissociate antiprogestational from antiglucocorticoid activity have been limited to modifications of existing steroidal antiprogestins. Unfortunately, a selective steroidal antiprogestin has not yet emerged. We believe that progress in this area has been limited by the approach that

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Address all correspondence and requests for reprints to: D. P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical School, Box 3813, Durham, North Carolina 27710. E-mail: McDon016@acpub.duke.edu.
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has been used in the past to screen for dissociated antiprogestins. Typically, *in vitro* receptor binding assays, assessing PR/GR selectivity, have been used to guide medicinal chemistry. This approach has not yet yielded a dissociated antiprogestin as it has been found that most compounds that display a reduced GR binding activity exhibit a commensurate decrease in affinity for PR (10). This observation suggested that a more predictive screen for novel antiprogestins was needed, one that did not discriminate based on receptor binding affinity, but rather on the ability of a compound to differentially affect PR or GR signaling.

Much of the justification for a mechanism-based approach to develop dissociated antiprogestins has come from our previous studies on the mechanism of action of PR agonists and antagonists (15–18). In these earlier studies, we identified two classes of antiprogestins that interact with similar, though distinct, regions within the PR ligand binding domain, resulting in unique alterations in PR structure (18). Subsequently, it was determined that members of one class of antiprogestins identified exhibited pure antiprogestenic activity in all contexts examined, whereas members of the second class functioned as antiprogestins in most contexts but had the ability to function as partial agonists in others (18). A potential molecular explanation for the differential activity of these two classes of antagonists was revealed when it was determined that the pure antiprogestins permitted the formation of high affinity interactions of PR with the nuclear receptor corepressors SMRT and NCoR, whereas the tissue selective antiprogestins (mixed agonists) formed weak associations with the same proteins. Importantly, overexpression of either corepressor had a pronounced effect on the activity of the PR mixed agonists where complete suppression of the partial agonist activity of these compounds was achieved. Cumulatively, these findings indicated that although the two classes of antiprogestins displayed similar PR binding affinities, they were mechanistically different. Based on this observation, which established a link between PR structure and biological activity, we considered that it may be possible to identify compounds that interact with both PR and GR but may not affect the transcriptional activity of these receptors in a similar manner. Therefore, in this study we used a series of mechanism based approaches to screen libraries of high affinity steroidal antiprogestins for compounds with reduced antiglucocorticoid activity.

Materials and Methods

Alkaline phosphatase assay

T47D cells were seeded into 96-well plates at a density of 10,000 cells/well in RPMI media supplemented with 10% FCS. Following a 24-h incubation, the cells were washed and fresh medium containing 2% FCS and ligand (10^{-6}–10^{-9} M) was added. The treated cells were incubated with ligand for 48 h, washed, and fixed with 5% formalin at room temperature for 30 min. Cells were subsequently washed and assayed for alkaline phosphatase activity as described previously (18, 19).

Mammalian transfections and luciferase assays

HeLa and T47D cells were maintained in MEM and RPMI supplemented with 10% FCS, respectively. Cells were plated in 24-well plates, 24–48 h before transfection. HeLa cells were transiently transfected for 3 h with a total of 3 μg of DNA per triplicate using Lipofectin. T47D cells were similarly transfected with Lipofectin for 2 h. After transfection, the cells were immediately washed and incubated with the designated ligands for 24 or 48 h. The cells were then lysed and analyzed for luciferase and β-galactosidase activity as previously reported (20).

Cell viability

CEM-C7 cells were maintained in RPMI 1640 media containing 10% dialyzed, heat-inactivated FBS. Cells were seeded at 1×10^5 to 3×10^5 cells per ml in 6-well plates and incubated with the designated ligands for 72 h. Following the incubation, 500 μl of cells were removed and the number of viable cells was assayed using trypan blue exclusion.

Relative binding affinities

All procedures were performed using a Biomek 1000 automated workstation (Beckman Coulter Instruments, Inc., Fullerton, CA). Tenfold serial dilutions (10^{-6}–10^{-10}) of the compound to be tested were prepared in a 10 mM Tris (pH 7.6) 0.3 MKCl, 5 mM DTT solution. A 100 μl aliquot of each dilution was transferred to a polystyrene tube containing 5 nM [^{3}H] progesterone or [^{3}H] dexamethasone (Amersham, Arlington Heights, IL). To each tube either PR containing extracts from baculovirus (20 μg total protein) or GR containing extracts from MDA-231 cells (250 μg total protein) were added and incubated overnight at 4 C. Hydroxylapatite slurry (100 μl) in 10 mM Tris (pH 7.6) and 2 mM DTT were added and the tubes were incubated for an additional 30 min at 4 C, after which they were centrifuged to recover the pellets. Hydroxylapatite pellets were washed four times with 1% Triton X-100, 10 mM Tris (pH 7.6), 5 mM DTT after which they were resuspended in 800 μl Ecoscint A scintillation fluid (National Diagnostic, Manville, NJ), and the activity was measured on a LS60001C scintillation counter (Beckman Coulter Instruments, Inc., Fullerton, CA).

Immunohistochemistry

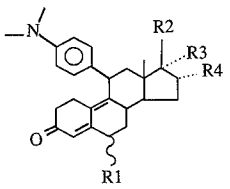
The subcellular distribution of human GR transiently transfected into COS-1 cells has been previously described (21). Briefly, COS-1 cells (African Green Monkey Kidney, ATCC) were grown in DMEM (Gibco BRL, Gaithersburg, MD) containing 9 mg/ml glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, and supplemented with 2 mM glutamine and 10% of a 1:1 mixture of FCS/calf serum (FCS:CS) (Irvine Scientific, Santa Ana, CA). Cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2. The cells were passed every 3–4 days and were maintained in culture for no longer than 15 passages. Cells were transfected by the commercial agent DMRIE C (Gibco BRL) as per manufacturer's instructions. Cells were incubated with the appropriate DNA/DMRIE C mixture for 4 h and placed in DMEM supplemented with steroid-stripped FCS:CS and further incubated at 37 C for 24 h. Transfected cells were then placed in two-chamber glass slides and incubated for an additional 24 h and then treated with 100 nM hormone or vehicle for 1 h. Cells were fixed and processed for immunohistochemical staining as previously described (21).

Results

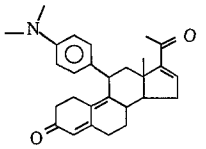
PR ligands can be classified into either of three mechanistically distinct groups

As an initial step in this study, we screened a series of steroidal PR ligands to identify compounds that displayed agonist, antagonist, or mixed agonist activity on PR. It was anticipated that this would allow the identification of mechanistically unique PR antagonists that could function as dissociated antiprogestins or which could serve as leads for additional synthetic chemistry. The structures of the compounds evaluated in this study are shown in Table 1. Previous studies with these compounds indicated that they could be separated into one of three groups based on how they interacted with PR (18). In this study, we evaluated whether the biological activity of these compounds reflected these mechanistic classifications. This was accomplished by evaluating each compound for agonist and antagonist ac-

TABLE 1. Structures of PR agonists and antagonists



RU486, 1-3,5-7



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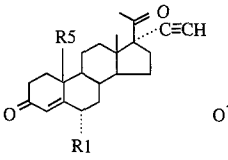

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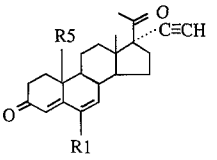

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Compound	R1	R2	R3	R4	R5
RU486	-H	-OH	$-C\equiv C-CH_3$	-H	-
1. 3021-002	-H	-OH	$-CH_2N_3$	-H	-
2. 3021-003	-H	-OH	$-CH_2OCH_3$	-H	-
3. 3021-012	-H	$-COCH_3$	-OAc	-H	-
4. 3021-023	-	-	-	-	-
5. 3021-020	$-\alpha CH_3$	$-COCH_3$	-H	$-C_2H_5$	-
6. 3021-021	$-\beta CH_3$	$-COCH_3$	-H	$-C_2H_5$	-
7. 3021-022	-H	$-COCH_3$	-H	$-C_2H_5$	-
8. 2207-222	-H	-	-	-	-H
9. 2207-225	$-CH_3$	-	-	-	$-CH_3$
10. 2207-226	$-CH_3$	-	-	-	$-CH_3$

tivity in PR-containing T47D cells on the endogenous progesterone-responsive alkaline phosphatase gene (19). Although the alkaline phosphatase gene is regulated by PR, it is not clear if this activity occurs in a direct or an indirect manner. As observed in Fig. 1A, progesterone administration induced significant alkaline phosphatase activity in this cell system. Compounds that, based on their effect on PR structure, were predicted to function as antagonists [RTI 3021–002 (RTI-002), RTI 3021–003 (RTI-003), and RTI 3021–012 (RTI-012)], exhibited no measurable agonist activity. Conversely, compounds that interacted with PR in a manner similar to progesterone [RTI 2207–222 (RTI-222), RTI 2207–225 (RTI-225), and RTI 2207–226 (RTI-226)] functioned as agonists. The PR ligands, RTI 3021–020 (RTI-020), RTI 3021–021 (RTI-021), and RTI 3021–022 (RTI-022), which induce unique structural alterations within the receptor, exhibited partial agonist activity in this assay, a result that distinguished them from agonists and antagonists. The classification of these compounds as partial agonists, as distinct from weak agonists, was confirmed by examining their ability to inhibit progesterone induced expression of alkaline phosphatase activity. As shown in Fig. 1B, the pure antagonists all functioned as potent PR antagonists and quantitative inhibition was achieved at concentrations as low as 100 nM. The partial agonist activity of RTI-020, -021, and -022 was confirmed by demonstrating that they inhibit progesterone activated PR transcriptional activity to a level equivalent to their maximal agonist activity. Although the direct measurement of alkaline phosphatase activity indicated that like progesterone, RTI-222, -225, and -226 function as PR agonists they may not function in an identical manner to progesterone in this assay. Specifically, it is noted that the maximal efficacy of the RTI agonists is significantly less than progesterone (Fig. 1A). Paradoxically, these compounds do not inhibit progesterone agonist activity when tested in the antagonist mode. As yet, we have been unable to explain this result. As shown below, however, additional experiments indicate that this particular activity of the RTI agonists may be unique to the alkaline phosphatase promoter.

It has previously been determined that the activity of the ER-mixed agonist tamoxifen is influenced by cell and promoter context (22, 23). In light of this, we decided to examine whether or not the partial agonist activity of RTI-020, -021, and -022 was likewise affected by the context in which it was assayed. To address this issue we evaluated the pharmacology of the PR-mixed agonists on a transfected MMTV promoter in PR-containing T47D cells and compared it to that of the pure agonists and antagonists. As observed in Fig. 2A, the pure antagonists and agonists functioned predictably. However, in this environment RTI-020, -021, and -022 do not exhibit measurable agonist activity (Fig. 2A) and at 100 nM all members of this class functioned as efficient antagonists of progesterone agonist activity (> 95% efficacy; Fig. 2B). Similar results were obtained in transfected CV-1 cells using the same experimental paradigm (data not shown). Taken together, these results confirmed that PR ligands could be separated into at least three functionally distinct classes. We proceeded, therefore, to assess the antiglucocorticoid activity of these newly identified PR antagonists and partial antagonists.

The compounds RTI-022 and RTI-012 differ in their ability to modulate PR and GR transcriptional activity

In the past, it was generally held that the ability of a nuclear receptor antagonist to inhibit transcriptional activity was determined solely by its affinity for its cognate receptor (1). If this were true, then binding selectivity would be the only way of generating pure antiprogestins that were GR sparing. It is becoming more apparent, however, that the effect of the

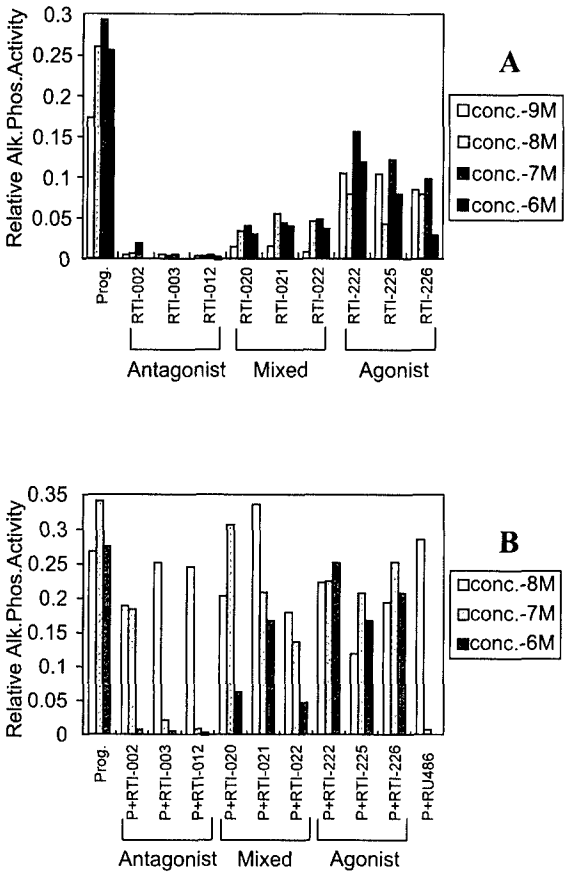

FIG. 1. Progesterone receptor ligands can be divided into three classes: agonists, mixed agonists and antagonists. The agonist and antagonist activities of the RTI series of PR ligands (the structures shown in Table 2) were assessed on the progesterone responsive alkaline phosphatase gene in T47D cells. T47D cells were incubated with the indicated ligands (A) alone to assay for agonist activity (10^{-6}–10^{-9} M) or (B) together with progesterone (10^{-7} M) to assay for antagonist activity (10^{-6}–10^{-8} M). After 48 h incubation with ligand, the cells were fixed and assayed for alkaline phosphatase activity. Each data point represents the average of triplicate determinations.

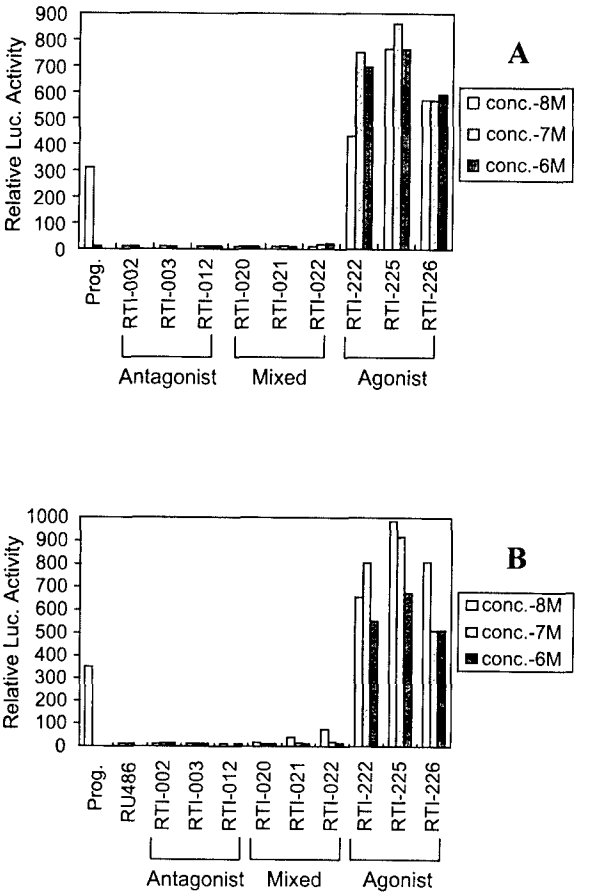

FIG. 2. PR mixed agonist activity is promoter dependent. The agonist and antagonist activity of a series of PR ligands was analyzed in PR-containing T47D human breast cancer cells that were transiently transfected with an MMTV-Luciferase reporter plasmid and a CMV-β-galactosidase expression plasmid for normalization. To assay agonist activity, transfected cells were incubated with (A) either 10^{-8} M progesterone or increasing concentrations of the indicated ligands (10^{-6}–10^{-8} M). Antagonist activity (B) was assessed by incubating cells with either 10^{-8} M progesterone alone or together with increasing concentrations of competing ligands as indicated (10^{-6}–10^{-8} M). Forty-eight hours post transfection, the cells were lysed and assayed for luciferase and β-galactosidase activities. The data points are averages of triplicate determinations.

ligand on overall receptor structure is an equally important determinant of biological activity. This has led to the concept that antagonists are "actively" involved in inhibiting receptor action (15, 18, 24, 25). If this model is correct, then binding affinity and antagonistic activity are not necessarily equivalent. The availability of a repertoire of novel, mechanistically different antiprogestins provided us with the reagents to test this model. For these specific studies, the pure antagonist RTI-012 and the mixed agonist RTI-022 were chosen for an analysis of their ability to inhibit GR transcriptional activity. These specific ligands were selected because they exhibit similar relative binding affinities (RBA) for both PR and GR, allowing a direct analysis of the role of "mechanism" in determining the relative GR/PR cross-reactivity of a PR ligand (Table 2). When compared with dexamethasone, it was observed that RTI-022, RTI-012, and RU486 (the standard used in our assays) had similar GR binding affinities (RBAs 5.7, 5.2, and 13.9, respectively) to the pure agonist dexamethasone. A similar analysis comparing the affinities of these compounds for PR indicated that, compared with progesterone, the RBAs for RTI-012, RTI-022, and RU486 were 12.7, 11.9, and 6.8, respectively. Thus, if ligand binding is the primary determinant of antagonist efficacy, then these compounds should display equivalent antiprogestenic and antiglucocorticoid activities. To test this hypothesis, we compared the ability of RU486, RTI-012, and RTI-022 to inhibit PR and GR transcriptional activity in transfected cells.

To assess the antagonist activity of RTI-012, RTI-022, and RU486, we transfected the PR/GR responsive reporter gene MMTV-LUC into T47D cells and assayed the ability of these compounds to inhibit the agonist activity of the synthetic progestin R5020. The results of this analysis, shown in Fig. 3, demonstrate that all three compounds are effective PR antagonists. In accord with the observed affinity differences, we noticed that the antagonist potency of RU486 was slightly greater than either of the two RTI compounds, which themselves behaved quite similarly in this assay. Thus, in this cell and promoter context, the *in vitro* PR binding affinity of these compounds and their PR antagonist efficacy match closely.

TABLE 2. Receptor binding characteristics

Analog	PR-A		GR		PR-RBA/GR-RBA[a]
	K_d (nM)	RBA	K_d (nM)	RBA	
Dexamethasone			9.5 ± 1.5	1	1
Progesterone	3.95 ± 0.25	1			1
RU486	0.58 ± 0.03	6.8	0.68 ± 0.06	13.9	0.48
RTI-012	0.31 ± 0.04	12.7	1.68 ± 0.18	5.7	2.3
RTI-022	0.33 ± 0.03	11.9	1.83 ± 0.16	5.2	2.2

Data shown as mean ± SEM (N = 2). [^{3}H] Progesterone was used as the ligand for PR and [^{3}H] dexamethasone for GR. Prep was Baculovirus extracts of PR-A and cytosolic extract from MDA-231 cells for GR. Total protein per tube was 20 μg and 250 μg for PR and GR, respectively. Incubation overnight at 4 C.

RBA, Relative binding affinity, progesterone and dexamethasone = 1.

[a] Ratio of RBA at progesterone receptor to RBA at glucocorticoid receptor. Values >1 favor affinity for progesterone receptor over glucocorticoid receptor.

Similar results were observed in HeLa cells in which PR and a PR-responsive promoter were cotransfected (data not shown).

We next performed a comparison of the ability of the selected compounds to inhibit GR transcriptional activity. This was accomplished by cotransfecting GR and the GR/PR responsive MMTV-LUC reporter gene into HeLa cells and assessing the ability of these compounds to inhibit dexamethasone-stimulated GR transcriptional activity. The results of this analysis are shown in Fig. 3B. As expected from its GR-binding affinity, RU486 functioned as an effective GR antagonist. Quite surprisingly, however, RTI-012 and RTI-022, whose affinities for GR were similar to each other and to that displayed by RU486, did not function as potent GR antagonists. Specifically, under the conditions of this assay the antagonist potencies of RU486 and RTI-012 differed by over 100-fold, whereas a greater than 1000-fold difference in potency was observed between RU486 and RTI-022. The differences between RTI-012 and RTI-022 may relate to subtle mechanistic differences between these compounds. Alternatively, it is possible that RTI-012 is converted to its 17α-OH metabolite, a transformation that would not express itself in the *in vitro* binding assays and may enhance its receptor binding affinity. These informative results indicated that, with respect to GR antagonism, there was a large discrepancy between GR antagonist efficacy and binding affinity. It must also be mentioned that neither RTI-012, nor RTI-022 exhibited any GR agonist activity when assayed on a GR-responsive promoter in transfected mammalian cells (data not shown).

RTI-012 and RTI-022 efficiently promote the interaction of PR, but not GR, with target gene promoters in vivo

We considered that one reason for the difference in GR antagonist efficacy manifest by RU486, RTI-022, and RTI-012 was that they were not equally effective at delivering GR to DNA. This possibility was tested by assessing the ability of these compounds to activate transcription of a GR-VP16 fusion plasmid. In this assay, GR/ligand complexes that bind DNA permit the activation of transcription by the VP16 activation domain contained within the chimeric GR. This approach was chosen as we and others have shown that the VP16 activation function, when used in the context of a receptor chimera, permits both agonists and antagonists to activate transcription upon DNA binding (22, 26). Thus, antagonists will function as agonists if they can deliver the chimera receptor to DNA. For this analysis, HeLa cells were transiently transfected with an expression vector encoding the GR-VP16 chimera together with one of two different GRE containing luciferase reporter vectors, MMTV-LUC or PRE-TK-LUC. As shown in Fig. 4A, both dexamethasone and RU486 efficiently delivered GR-VP16 to DNA. Interestingly, this is not the case when the assay is performed in the presence of either RTI-012 or RTI-022. Under the conditions of this assay, using saturating concentrations of test compounds, we observed that the GR/DNA binding activity of RTI-012 and RTI-022 was only 35% and 6%, respectively, of that observed in the presence of RU486 when assayed on the MMTV-LUC promoter. A similar result was observed when the assay was performed on the PRE-TK-LUC promoter. For comparative purposes, we performed the same type of assay using PR-VP16. The results of this analysis shown in Fig. 4 indicate that both RTI-022 and RTI-012 are capable of inducing high affinity PR-DNA interactions in a manner that is indistinguishable from RU486. Thus, we conclude from these results that the inability of RTI-012 and RTI-022 to efficiently deliver GR to DNA may explain their relatively weak GR-antagonist activity.

RTI-022 and RTI-012 differ from RU486 in their ability to efficiently induce nuclear translocation of GR

The results outlined above (Fig. 4) demonstrated that there were differences in the ability of antagonists to promote GR target promoter associations (RU486[tmt]RTI-012 > RTI-022). One explanation for this activity is that there were differences in the ability of these compounds to promote nuclear translocation. GR is unique among the nuclear receptors in that it resides in the cytoplasm of target cells in the absence of ligand (21). Upon binding an agonist such as dexamethasone, the receptor translocates to the nucleus where it exerts its regulatory activities (21). To test the effect of the RTI compounds on GR nuclear translocation we transfected COS-1 cells with an expression vector for GR and examined the cellular localization of the recombinant receptor using immunohistochemical techniques following treatment of the cells with selected agonists and antagonists. The results of this analysis are shown in Fig. 5. Both dexamethasone and RU486 promoted an efficient translocation of GR to the nuclear compartment of these cells. However, under the conditions of this assay both RTI-012 and RTI-022 were only partially active in this regard. We therefore concluded that RTI-012 or RTI-022 function predominantly as compet-

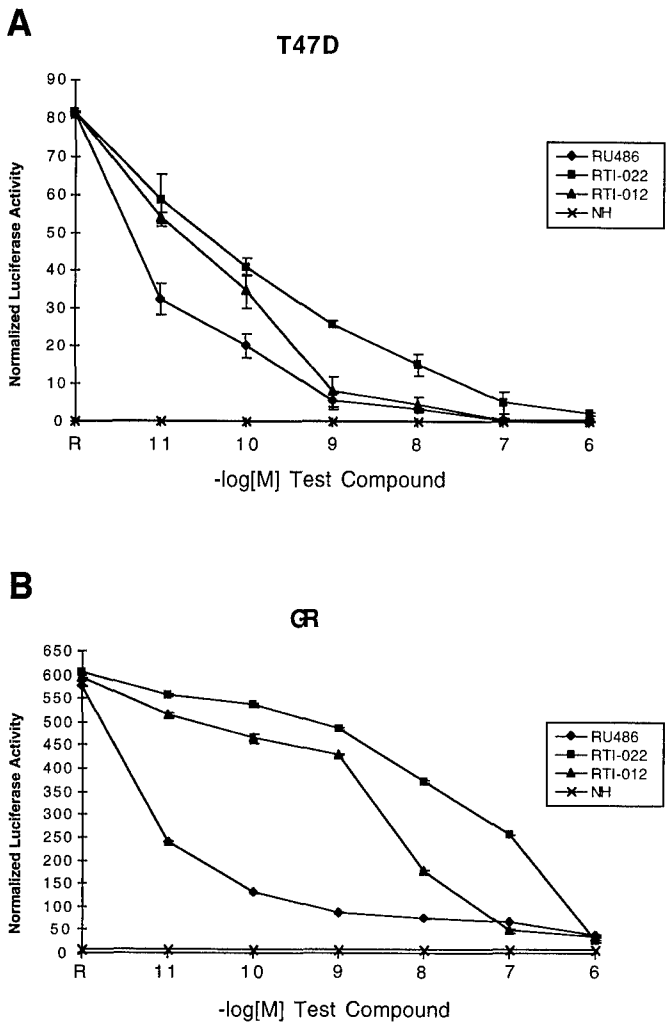

FIG. 3. RTI-012 and RTI-022 are potent antiprogestins that demonstrate weak antiglucocorticoid activity. A, The relative PR antagonist activity of RU486, RTI-012, and RTI-022 were compared in PR-containing T47D human breast cancer cells that were transiently transfected with an MMTV-Luciferase reporter plasmid and a CMV-β-galactosidase expression plasmid for normalization. To assay agonist activity, transfected cells were incubated with 10^{-8} M R5020 and increasing concentrations of the indicated antagonists (10^{-6}–10^{-11} M). Forty-eight hours post transfection, the cells were lysed and assayed for luciferase and β-galactosidase activities. The data points are averages of triplicate determinations. B, Antiglucocorticoid activity was analyzed in HeLa cells transiently transfected with a GR expression plasmid, the MMTV-Luciferase reporter plasmid, and a CMV-β-galactosidase plasmid for normalization. After transfection cells were treated with 10^{-9} M Dexamethasone (Dex) alone or in the presence of competing ligand as indicated (10^{-11}–10^{-6} M) for 48 h. Cells were lysed and assayed for luciferase and β-galactosidase activities. Each data point presented is the average of triplicate determinations.

itive antagonists on GR because the resulting receptor-ligand complexes cannot translocate efficiently to the nucleus and compete for DNA binding with agonist activated receptor.

RTI-022 exhibits weak antiglucocorticoid activity in GR-mediated apoptosis

The ability to develop compounds that effectively inhibit PR transcriptional activity but which do not inhibit GR actions is likely to facilitate the use of antiprogestins for the

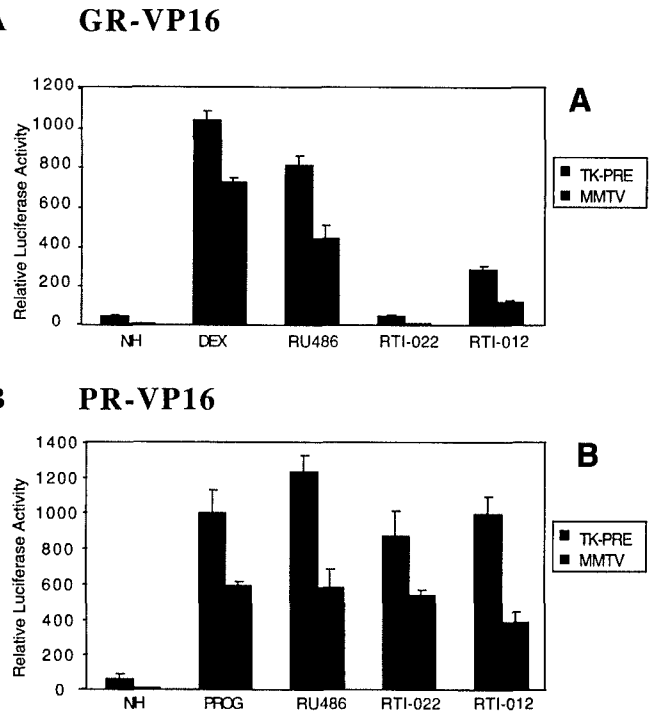

FIG. 4. RU486, RTI-022, and RTI-012 differ in their ability to facilitate interactions of PR and GR with their cognate target gene promoters. DNA binding ability was assayed by measuring the transcriptional activity of GR or PR fused to the VP16 activation domain on the PRE-containing luciferase reporter vectors MMTV-Luc or PRE-TK-Luc as indicated. For this analysis HeLa cells were transiently transfected with an expression vector for either (A) GR-VP16 or (B) PR-VP16, in combination with either a PRE-TK-Luc, or MMTV-Luc and a CMV-β-galactosidase normalization plasmid. Cells were incubated with dexamethasone (Dex), R5020, RU486, RTI-022, or RTI-012 (10^{-7} M) for 48 h followed by lysis and analysis for luciferase and β-galactosidase activities. Each data point represents the average of triplicate determinations. *Error* is represented as (±SEM).

treatment of several chronic diseases where inhibition of PR action is implicated. The molecular data presented thus far suggest that the RTI-012 and RTI-022 compounds may, if their pharmaceutical properties permit, be clinically useful compounds. To develop this hypothesis further we extended our studies to cell based models that may be more reflective of *in vivo* biological responses. We chose to use RTI-022 for these studies as it gave the largest separation between PR and GR antagonist activities and consequently would likely be the compound of choice for clinical development. Glucocorticoid agonists are effective in causing apoptosis in T-lymphoblasts, such as the human T-lymphoblastic cell line, CEM-C7, an event that is blocked by the antagonist RU486 (27). While GR transrepression of AP-1 activity has been implicated in Jurkat cells (28), GR-mediated up-regulation of GR and c-*jun* appears to regulate apoptosis in CEM-C7 cells (29, 30). Furthermore, suppression of GR-agonist induced up-regulation of c-*jun* gene expression using an antisense c-*jun* expression vector blocks the apoptotic response (30). We were interested, therefore, in assaying the ability of RTI-022 to prevent dexamethasone (Dex) induced apoptosis in this cell line. For this assay, CEM-C7 cells were grown in the presence of either vehicle, or dexamethasone alone, or together with increasing concentrations of the designated li-

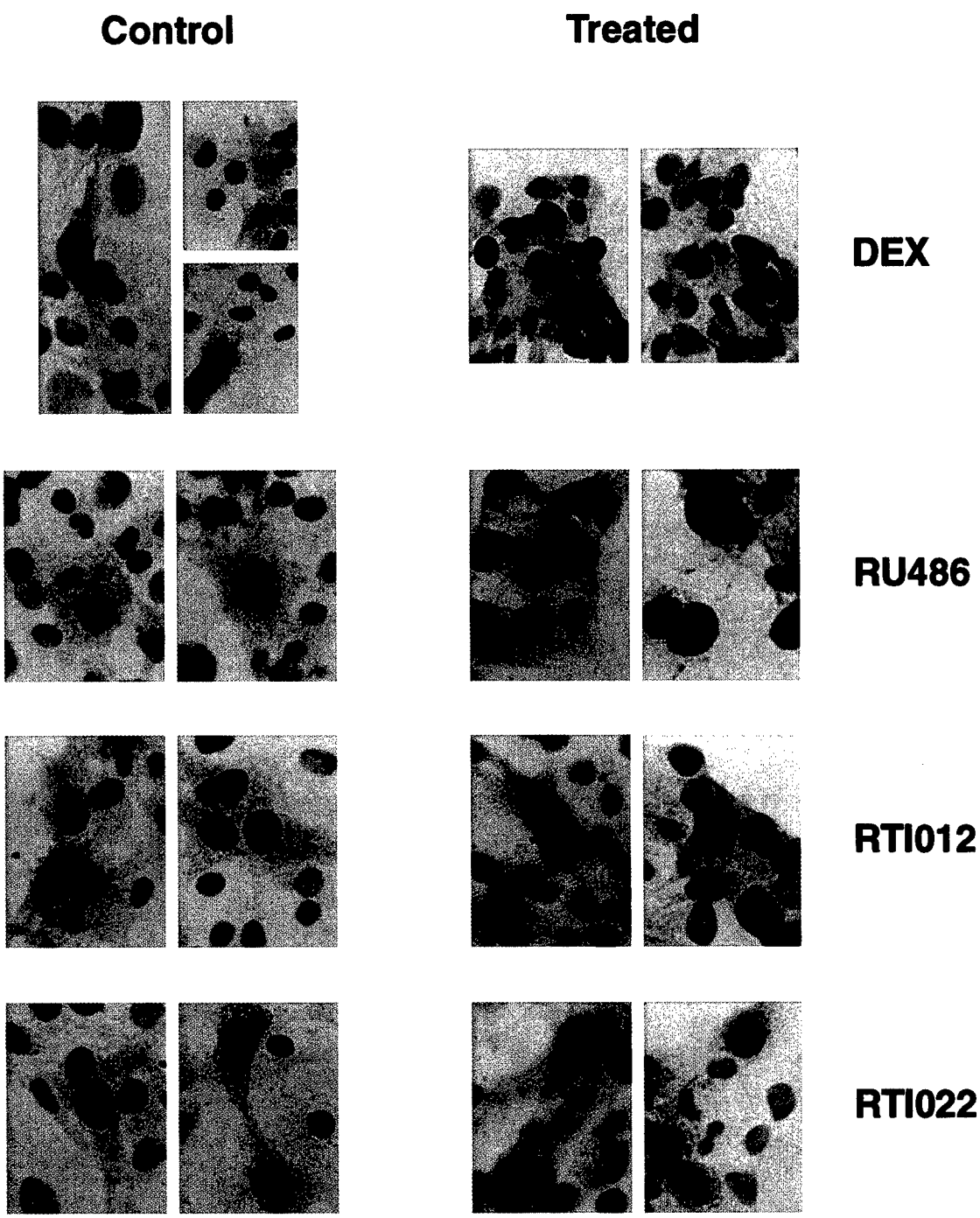

FIG. 5. Nuclear translocation of glucocorticoid receptor in the presence of RU486, RTI-022, or RTI-012. Wild-type human glucocorticoid receptor (hGR) complementary DNA was transiently expressed in COS-1 cells and treated with 100 nM hormone (Treated) or not (Control) for 2 h. Cells were fixed and subsequently incubated with an epitope purified GR specific antibody. Immunoreactivity was visualized using an avidin-biotin peroxidase stain. Photomicrographs were taken and then evaluated in a blind manner at 600× magnification using Kodak Royal Gold ASA-200.

gands, after which cell viability was measured using trypan blue exclusion. The negative control, progesterone, which exhibits a much lower affinity for GR (30 nM) (Cook, C. E., data not shown) than the compounds we are investigating, did not prevent dexamethasone from inducing apoptosis (Fig. 6). As previously reported, RU486 completely prevented dexamethasone-induced apoptosis when these compounds were added in equimolar concentrations and gave 50% protection when added at a concentration 1/10th that of dexamethasone. This is the expected result given that RU486 has nearly a 13-fold higher affinity for GR than does dexamethasone. Interestingly, when assayed under the same conditions RTI-022, whose affinity for GR is only 2.5-fold less than RU486, required 50–100 times more compound to evoke the same response. Cumulatively, therefore, our data, emanating from both cotransfection and cell based assays, indi-

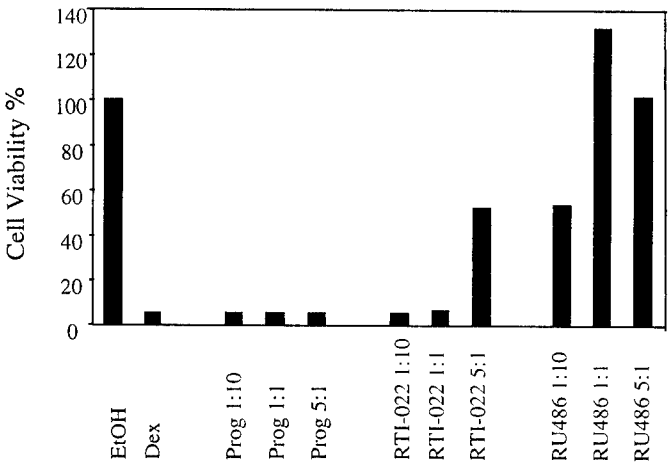

FIG. 6. RTI-022 is not a potent inhibitor of dexamethasone induced apoptosis. CEM-C7 cells were seeded at 300,000 cells/ml and grown in the presence of vehicle (EtOH), 1 μM dexamethasone, or 1 μM dexamethasone (Dex) with increasing concentrations (0.1, 1, or 5 μM) of RU486, progesterone (Prog) or RTI-022 (1:10, 1:1 and 5:1) molar ratios compared with dexamethasone (Dex). Following a 72-h incubation, cell viability was measured using trypan blue exclusion. The data are presented as % of viable cells remaining following ligand treatment compared with vehicle alone.

cate that PR antagonists can be developed that do not significantly impact GR signaling.

Discussion

Classical receptor theory predicts that the biological activity of an agonist, or an antagonist, is a reflection of its affinity for its target receptor (1). However, it is clear that ligand binding affinity is only one of many factors that influence the pharmacology of steroid receptor ligands (22, 31, 32). For instance, the high affinity ER-ligand tamoxifen can function as an ER-antagonist, partial agonist or a full agonist, depending on the cell context in which it is analyzed (23, 33). These data suggest that ER is not functioning in an identical manner in all cells. This concept appears not to be restricted to ER because we have recently determined that PR ligands can be classified into three distinct groups, pure agonists, mixed agonists or pure antagonists, and that the relative agonist/antagonist activities of the mixed agonists is determined, to a large extent, by the cell and promoter context in which transcriptional activity is assessed (18). Cumulatively, these studies on the molecular pharmacology of ER and PR suggest to us that it may be possible to use mechanism based approaches to discover novel steroid receptor ligands that display improved selectivity over existing compounds.

In this study, we undertook a molecular approach to understand the mechanism by which antiprogestins manifest antagonist activity on PR and GR. The currently available antiprogestins also function as effective antiglucocorticoids (13, 14). Thus, for applications that require chronic administration there is a medical need to develop dissociated antiprogestins; compounds that display no or reduced antiglucocorticoid activity (9). However, there has been little success in identifying antiprogestins that do not function as antiglucocorticoids (10, 13, 14). This may relate to the fact that the currently available antiprogestins are steroidal, derived from the same chemical backbone, and so may function by very similar mechanisms (13). The recent identification of a new class of PR mixed agonists, which interact with the PR hormone binding domain in a distinct manner, prompted us to reexamine the issue of GR cross-reactivity of PR antagonists (18). In this study, we profiled this new series of PR ligands and determined that the compounds RTI-022 and RTI-012 that functioned as potent PR antagonists *in vitro* exhibited significantly less GR antagonist activity than their receptor binding affinities would predict. To understand the discrepancy between binding affinity and biological potency, we compared the ability of RU486, RTI-022, and RTI-012 to facilitate the interaction of GR with target gene promoters. These studies revealed that neither RTI-012 or RTI-022 were as effective as RU486 at inducing nuclear translocation of GR. In contrast, however, RTI-012, RTI-022 and RU486 efficiently facilitated PR/DNA interactions and demonstrated comparable progesterone antagonist activities. Thus, although we previously had shown that RTI-012 and RTI-022 interact with different regions of the PR-ligand binding domain and do not inhibit PR-transcriptional activity in the same manner, they both efficiently delivered PR to DNA indicating that it was post DNA binding events that distinguished these compounds. When assayed on GR, we were surprised to find that RTI-012 and RTI-022, that displayed high affinity GR binding, were not potent antagonists. This was in great distinction to RU486, an affinity matched ligand, which functioned as a potent PR and GR antagonist. Thus, although we can classify compounds as PR agonists, antagonists or partial agonists based on how they interact with PR, these classifications do not predict the likely GR cross-reactivity of specific compounds. Thus, at this point, we believe that the unique chemical structures of RTI-012 and RTI-022 have some effect on GR that distinguishes them from RU486. This interesting possibility will be followed up in subsequent studies. Regardless, these data strongly support our hypothesis that binding affinity alone is not sufficient to predict the biological activity of a receptor antagonist.

The studies presented here, and those of others, are compatible with the existence of two distinct types of antagonists, competitive and active antagonists (Fig. 7). Using GR antagonism as an example, we propose that the RTI compounds function only as competitive antagonists; a one-step process in which agonists and antagonists only compete for receptor binding. Possibly because of a specific conformational change, the resultant GR ligand complex does not enter the nucleus and therefore does not directly oppose the actions of residual agonist activated receptor. Because competitive inhibitors do not prevent agonist occupied receptors from binding DNA and activating transcription, their antagonist activity is governed mainly by affinity. In contrast to RTI-022 and RTI-012, RU486 functions as an active antagonist of GR transcriptional activity. Thus, RU486 not only competitively inhibits agonist binding to GR but permits the formation of a ligand-GR complex that can participate actively in the inhibition process. Specifically, these complexes can bind with high affinity to target gene promoters and block agonist activated receptor from interacting with its DNA-target site. In some contexts, members of this class of active antagonists can function as partial agonists; an event that can only occur

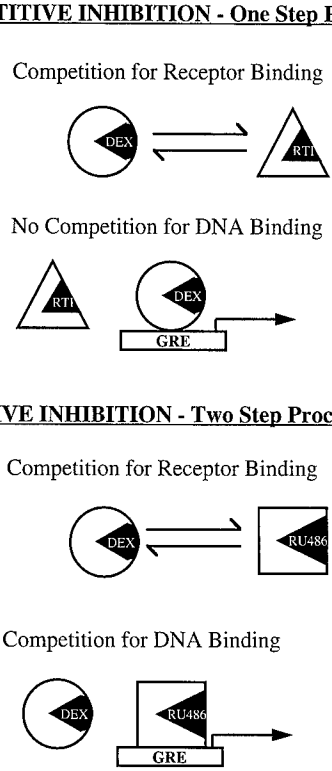

FIG. 7. Competitive *vs.* active inhibition of GR transcriptional activity. Competitive inhibition is a one-step process in which the antagonist competes with the agonist for receptor binding. Competitive inhibitors induce a conformational change in the receptor that is incompatible with DNA binding, preventing the antagonist occupied receptors from competing at the level of DNA binding. The two RTI compounds examined in this study thus function as competitive inhibitors of GR. Active inhibition is a two-step process in which 1) the antagonist competes with the agonist for receptor binding and 2) antagonist occupied receptors compete with agonist occupied receptors for binding to glucocorticoid responsive elements. The ability of PR and GR to recruit the transcriptional corepressors N-CoR and SMRT when occupied by active antagonists is likely to be important also. These proteins are part of a large complex that can de-acetylate histones H3 and H4 resulting in nucleosome condensation and transcriptional silencing.

when the receptor binds DNA. Indeed, Nordeen *et al.* (34) have demonstrated that RU486 can in fact manifest partial GR agonist activity in some contexts. Although the mechanism of this partial agonism remains to be determined, it is likely that differences in the expression of specific receptor associated proteins contribute significantly to the degree of agonist activity manifest by the RU486/GR complex. Therefore, in those contexts in which RU486 exhibits partial agonist activity, competitive inhibitors, at saturating doses, are more likely to function as pure antagonists. As a final note on this topic, we believe that because RU486, RTI-012, and RTI-022 efficiently deliver PR to DNA, they are functioning as active antagonists of PR transcriptional activity. This highlights the need to qualify the classification of a given compound with respect to a specific receptor.

One of the major findings of this paper is that, with respect to RTI-012 and RTI-022, there is a large discrepancy between the *in vitro* GR-binding affinity and antagonist potency. In the past, discrepancies of this nature were usually explained by differences in metabolism and/or pharmacokinetics; factors that are unlikely to be important in this case. However, we and others have defined a molecular mechanism that adequately explains active antagonism. Specifically, it has been determined that active antagonists like RU486 facilitate the interaction of PR and GR with the nuclear corepressors SMRT and N-CoR. The nuclear corepressors were originally identified as proteins that could bind to unoccupied TR and RAR located on target gene promoters and permit these receptors to function as transcriptional repressors (35, 36). Although the mechanism by which the corepressors exhibit their inhibitory activity remains under investigation, it appears that they are part of a multiprotein complex that is responsible for deacetylating histones H3 and H4 and facilitating a local condensation of chromatin (37–39). Recently we, and other groups, have been able to show that the influence of the corepressors is not restricted to the Class II nuclear receptors but that they are also an important part of PR, GR, and ER pharmacology (17, 40, 41). Specifically, it was shown that in the presence of pure antagonists, PR was capable of high affinity interactions with either N-CoR or SMRT (17, 41). Agonist binding abolished these interactions and partial agonists demonstrated an intermediate activity as expected (17). Thus, the model for active inhibition must be expanded to incorporate this new information. Specifically, an active antagonist such as RU486 can competitively bind to its target receptor, induce high affinity DNA binding and subsequently recruit an inhibitory complex that is capable of enzymatically altering chromatin structure. In support of this model, we have been able to show that the corepressor SMRT can interact with both PR and GR when activated by RU486 (17). Thus, it is likely that the reason why RTI-022 and RTI-012 function only as competitive antagonists of GR activity is that they are unable to translocate GR to the nucleus. Thus, the association of the receptor with the corepressor is prevented.

In summary, this work has led to the identification of RTI-022 and RTI-012, compounds that function as competitive antagonists of GR function and active antagonists of PR transcriptional activity. These mechanistic differences manifest themselves as a 1- to 400-fold discrepancy between binding and antagonist efficacy with respect to GR activity and comparable binding and antagonist potency on PR. Thus, a separation between GR and PR antagonism is afforded by virtue of differences in the mechanism of action of these compounds on the two different receptors. This result validates using a mechanism-based approach to develop dissociated antiprogestins that, when used in combination with traditional direct binding approaches, is likely to be a powerful combination in the discovery of dissociated antiprogestins. In addition to providing useful insights into the pharmacology of PR and GR, we believe that RTI-012 and RTI-022 will find use *in vivo*, both as research tools and hopefully as drugs where it is important to separate antiprogestenic from antiglucocticoid activities.

Acknowledgments

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Appendix #4

RECENT PROGRESS IN HORMONE RESEARCH, VOL. 54

The A and B Isoforms of the Human Progesterone Receptor: Two Functionally Different Transcription Factors Encoded by a Single Gene

PALOMA H. GIANGRANDE AND DONALD P. MCDONNELL

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

In humans, the biological response to progesterone is mediated by two forms of the progesterone receptor (hPR-A; 94kDa and hPR-B; 114kDa). These two isoforms are transcribed from distinct, estrogen-inducible promoters within a single-copy progesterone receptor (PR) gene; the only difference between them is that the first 164 amino acids of hPR-B are absent in hPR-A. In most cell lines, hPR-A functions as a transcriptional repressor of progesterone-responsive promoters, whereas hPR-B functions as a transcriptional activator of the same genes. The observation, made in the early 1990s, that shorter isoforms of some transcriptional activators can act as transrepressors of the transcriptional activity of the larger isoforms, initiated a line of investigation that led to the discovery that hPR-A is a strong transrepressor of hPR-B activity. Interestingly, hPR-A also functions as a transdominant repressor of the transcriptional activity of the estrogen, glucocorticoid, androgen, and mineralocorticoid receptors. A specific inhibitory domain (ID) within hPR-A responsible for this activity has been mapped to the extreme amino terminus of the receptor. Interestingly, although this inhibitory domain is contained within both PR isoforms, its activity is manifest only in the context of hPR-A.

The identification of a discrete inhibitory region within hPR-A, whose activity was masked in the context of hPR-B, suggests that these two receptor isoforms may interact with different proteins (transcription factors, co-activators, co-repressors) within the cell. In support of this hypothesis, we have recently observed that the co-repressor SMRT (silencing mediator of retinoid and thyroid receptors) interacts much more tightly with hPR-A than with hPR-B. This important finding led to the initial conclusion that the ability of hPR-A to repress hPR-B transcriptional activity could occur as a consequence of hPR-B/A heterodimerization, where the presence of SMRT in the complex could prevent transcriptional activation. The observation, however, that hPR-A also inhibits human estrogen receptor (hER) transcriptional activity, a receptor with which hPR-A is not able to heterodimerize, suggests that there must be additional complexity. This chapter outlines what is known about the mechanism of action of hPR-A and hPR-B and how this knowledge has enhanced our understanding of PR pharmacology.

I. Introduction

The steroid hormone progesterone is a key regulator of processes involved in the development and maintenance of the reproductive system (Clarke and Sutherland, 1990). The successful use of antiprogestins such as RU486 (Mifepristone)

in the treatment of diseases such as brain meningiomas, endometriosis, and uterine fibroids has also implicated progesterone as a regulatory hormone in a wide range of additional biological processes (Poisson *et al.*, 1983; Colletta *et al.*, 1991; Kettel *et al.*, 1991; Horwitz, 1992; Lundgren, 1992; Brandon *et al.*, 1993; Carroll *et al.*, 1993). Not surprisingly, therefore, there is a great deal of interest in defining the molecular mechanism of action of the progesterone receptor (PR), with a view to developing novel pharmaceuticals.

The progesterone receptor is a ligand-activated transcription factor. It belongs to the nuclear receptor superfamily of transcription factors that includes receptors for steroid hormones, thyroid hormone, vitamin D, and retinoids. Included in this family of receptors are the orphan receptors, for which ligands have not yet been identified (Mangelsdorf and Evans, 1995). All steroid receptors share a similar basic structure composed of 1) a highly conserved DNA-binding domain (DBD); 2) a hormone-binding domain (HBD) that is conserved among the related steroid receptors such as the PR, the glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR); 3) a hinge region located between the DBD and HBD; and 4) an N-terminal domain, which is the most variable region among the family members (Gronemeyer, 1991; Kastner *et al.*, 1995; Beato *et al.*, 1995, Gronemeyer and Laudet, 1995) (Figure 1).

Reflecting the similarity in their modular structure, the general mechanism of action of PR is similar to other members of the steroid receptor family (Figure 2) (McDonnell, 1995). In the absence of ligand, the receptor is transcriptionally

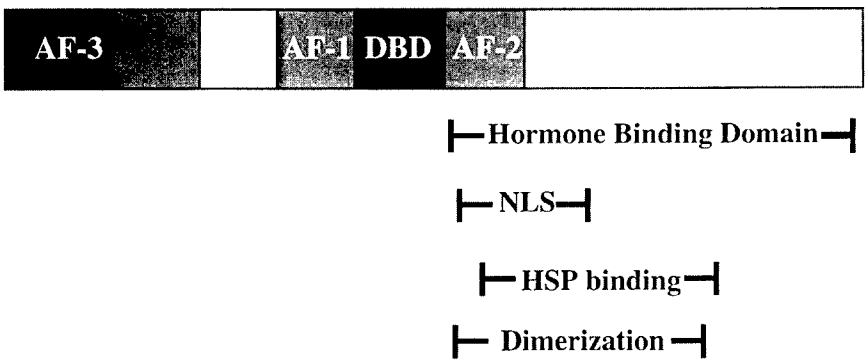


FIG 1. Progesterone receptor (PR) structure. The basic structure of the progesterone receptor is similar to that of other nuclear receptors. The progesterone receptor is composed of a highly conserved DNA-binding domain (DBD), a hormone-binding domain (HBD) conserved among the related steroid receptors, a hinge region located between the DBD and HBD, and an N-terminal domain, which is the most variable region among the family members. Within the HBD are regions responsible for receptor dimerization, interaction with heat-shock proteins (HSPs), nuclear localization, and ligand-dependent activation (activation function-2, AF-2). Two additional activation functions (AF-1 and AF-3) are located within the amino terminus of PR-B. AF-3, however, is absent in hPR-A.

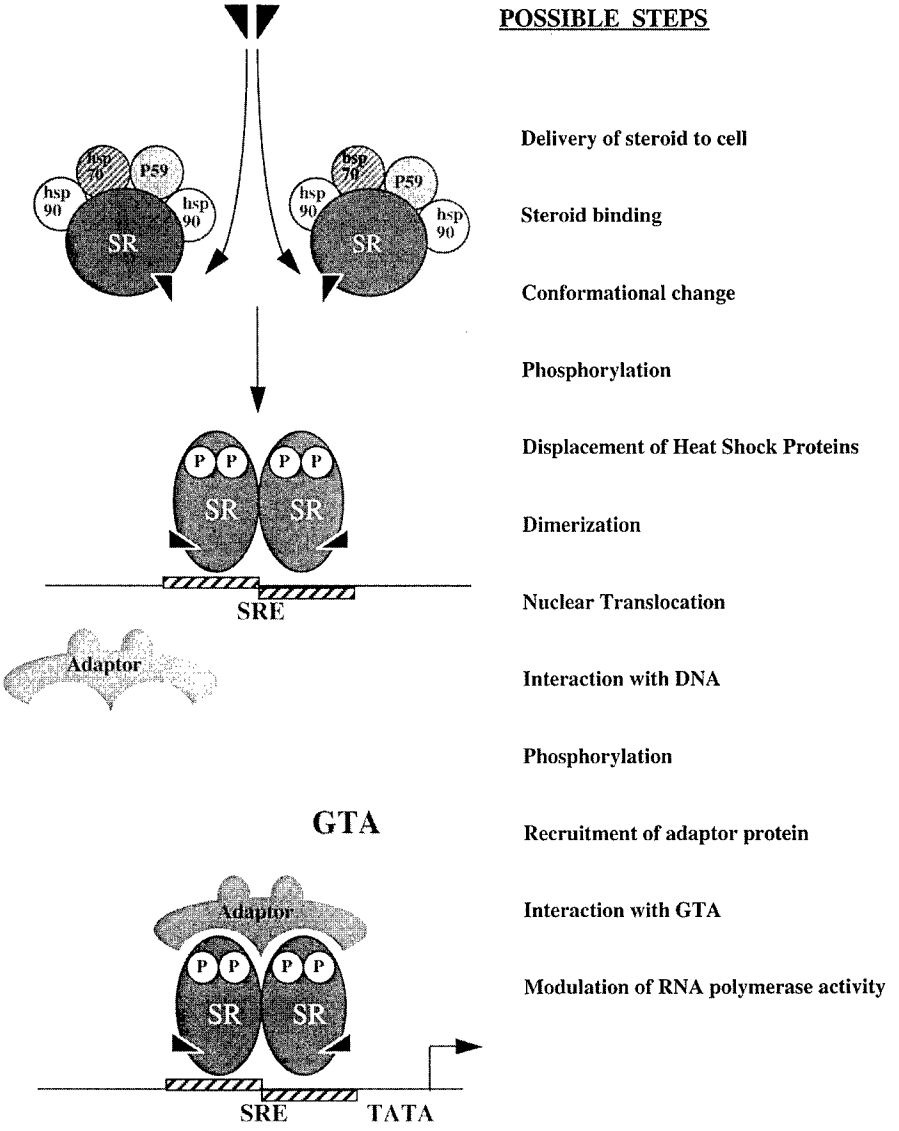


FIG 2. Progesterone receptor action. Simplified schematic of the steps involved in steroid receptor action. Upon ligand binding, the progesterone receptor undergoes a distinct conformational change, which is characterized by phosphorylation of the receptor, displacement of heat shock proteins, and receptor dimerization. Once in the nucleus, the dimerized receptor interacts with its cognate element on the DNA, where the phosphorylation state of the receptor is further enhanced. The DNA-bound receptor dimer recruits adaptor proteins that act as bridging factors with the general transcription apparatus (GTA). Interaction with the GTA results in steroid hormone receptor-mediated modulation of RNA polymerase activity.

inactive and remains in the nuclei of target cells, sequestered in a large complex with heat shock proteins (HSPs: HSP-90, HSP-70, and P59) (Beato *et al.,* 1987; Pratt, 1990; Picard *et al.,* 1990; Bagchi *et al.,* 1991; Smith and Toft, 1993). Upon ligand binding, however, the receptor undergoes a distinct change in conformation, which results in the dissociation of a monomeric receptor from the heat-shock complex (Allan *et al.,* 1992a,b; McDonnell *et al.,* 1995). The ligand-bound receptors then dimerize and bind to progesterone responsive elements (PREs) within the regulatory region of target genes (Beato *et al.,* 1987). The DNA-bound receptor can then either positively or negatively impact target gene transcription, an event that is influenced by both cell and promoter context. Genetic, biochemical, and pharmacological analysis of this latter step in the PR signal transduction pathway has revealed at least two mechanisms by which PR influences target gene transcription. Specifically, it has been determined that the ligand-activated receptor can contact components of the general transcription machinery (GTM) either directly or indirectly through receptor co-activator or adaptor proteins (Klein-Hitpass *et al.,* 1990; Ing *et al.,* 1992; Halachmi *et al.,* 1994; Oñate *et al.,* 1995; Cavaillès *et al.,* 1995; Le Dourin *et al.,* 1995; Hanstein *et al.,* 1996; Voegel *et al.,* 1996; Kamei *et al.,* 1996). These two different pathways of transcriptional activation are not mutually exclusive and are differentially utilized by PR in different cells, providing an explanation for the cell-selective activities that are manifested by different PR ligands. More importantly, this complexity suggests that it may be possible to develop pharmaceuticals that function as progestins or antiprogestins in a tissue-selective manner. Adding further to the complexity of the PR signal transduction pathway is the fact that PR exists in most species as two isoforms, PR-A and PR-B (Lessey *et al.,* 1983). This chapter specifically focuses on the role of both A and B isoforms in PR pharmacology and considers how this information will likely impact the development of novel progesterone receptor modulators.

II. Progesterone Receptor Isoforms and Their Role in PR Pharmacology

Human PR exists as two isoforms: hPR-B (114 kDa) and hPR-A (94 kDa) (Lessey *et al.,* 1983) (Figure 3). hPR-A is a truncated form of hPR-B, lacking the first 164 N-terminal amino acids. Both isoforms have been identified in most species, with the exception of the rabbit, where hPR-B alone has been detected (Loosfelt *et al.,* 1986). In humans, the two isoforms are transcribed from two distinct, estrogen-inducible promoters within a single-copy PR gene by alternative initiation of transcription (Kastner *et al.,* 1990; Gronemeyer, 1991). Under most circumstances, both hPR-A and hPR-B are present in target cells in equimolar amounts; however, differences in the relative expression level of these two isoform are also observed in some systems (Lessey *et al.,* 1983). Specifically, in the uterus, hPR-A:hPR-B ratios range from 50:1 to 2:1 during the menstrual cycle,

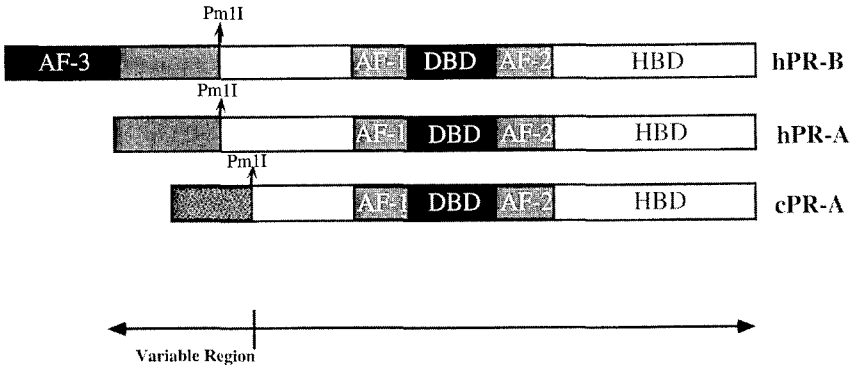


FIG 3. Sequence similarities among PR isoforms. The DNA sequences of the human and chicken isoforms of the progesterone receptor were obtained from GENBANK. Regions of amino acid similarities between hPR-A and cPR-A were determined using the DNA Strider and LALNVIEW programs. The regions of least homology are located upstream of the unique PmL I restriction site present in both receptors (55% similarity, 30% identity). Regions of high homology are found downstream of the PmL I restriction site (90% similarity, > 72% identity). The amino acid sequence of the B isoform of the human progesterone receptor is also detailed. hPR-B, human progesterone receptor-B; hPR-A, human progesterone receptor-A; cPR-A, chicken progesterone receptor-A; HBD, hormone-binding domain; AF-2, activation function-2; DBD, DNA-binding domain; AF-1, activation function-1; AF-3, activation function-3.

due mainly to increases in the expression level of hPR-B (Wiehle *et al.,* 1995), while low hPR-B levels (high A:B ratio) have been detected in primary breast tumors as well as endometrial cancers (Graham *et al.,* 1995,1996; Kumar *et al.,* 1998). One proven consequence of two forms of PR is the existence within the cell of three distinct chimeric states of hPR: A:A monomers, A:B heterodimers, and B:B monomers (DeMarzo *et al.,* 1991,1992). Since the A and B isoforms of hPR are not functionally equivalent, it is likely that the three dimeric forms of the activated receptor are also not functionally identical. Thus, differences in PR isoform expression are likely to influence cellular responsiveness to progestins and antiprogestins.

Initial work done on the biochemical properties of the human PR isoforms indicated that the two receptors had similar DNA- and ligand-binding affinities (Christensen *et al.,* 1991). It was not until the cDNAs for these receptors were cloned, however, that their different transcriptional activities were apparent. Specifically, work done using reconstituted progesterone-responsive transcription systems in various mammalian cells revealed that hPR-A and hPR-B have different promoter specificities (Vegeto *et al.,* 1993). In addition, the transcriptional activities of these isoforms were also shown to be dissimilar and to vary, depending on cell and promoter context (Tung *et al.,* 1993; Vegeto *et al.,* 1993;

Wen *et al.,* 1994; Chalbous and Galtier, 1994; McDonnell *et al.,* 1994). In most contexts examined, hPR-B functioned as an activator of reporter genes containing classical PREs, whereas the shorter hPR-A was transcriptionally inactive (Tung *et al.,* 1993; Vegeto *et al.,* 1993). Thus, the function of hPR-A in PR biology was, at that time, an enigma. However, as is usually the case in biology, there are lessons to be learned from other unrelated systems. Indeed, a review of the literature indicated that, in addition to hPR-A and hPR-B, there were several additional examples where two forms of a transcription factor existed within target cells as a consequence of alternate initiation of transcription or translation (Descombes and Schibler, 1991; Rentoumis *et al.,* 1990; Dobrzanski *et al.,* 1991; Foulkes and Sassone-Corsi, 1992). One of the most relevant with regard to the two isoforms of PR is the liver transcriptional activator LAP, which is co-expressed in most tissues with a shorter transcriptionally inactive form, LIP (Descombes and Schibler, 1991). LIP is generated by the use of an alternative translation start site within the LAP coding sequence. This event gives rise to an N-terminally truncated form of the protein that, when co-expressed with LAP, downregulates LAP transcriptional activity (Descombes and Schibler, 1991). The similarity in the manner by which LAP/LIP and the PR isoforms were derived prompted us several years ago to examine whether hPR-A could function as a modulator of hPR-B transcriptional activity. This analysis revealed that, in those cell contexts where hPR-A did not activate transcription, it could function as a strong, ligand-dependent, transdominant repressor of hPR-B activity (Vegeto *et al.,* 1993). A representative experiment illustrating this is shown in Figure 4. Thus, similar to LAP/LIP, hPR-A/B represents a pair of transcription factors with distinctly opposite activities. It was inferred from these results that the pharmacological response of a cell to progestins and antiprogestins would be determined, in large part, by the relative expression of the two receptor isoforms.

One of the interesting features of the steroid receptor family of transcription factors is that, although each is responsible for a distinct regulatory pathway and responds to structurally different hormones, they share a tremendous degree of functional similarity. It is not surprising, therefore, that several points of convergence or cross-talk between receptor signaling pathways systems have been defined. What *was* surprising, however, was the finding that hPR-A was a key mediator of this cross-talk. Specifically, it was observed that, in addition to modulating hPR-B transcriptional activity, hPR-A also functioned as a transdominant repressor of the transcriptional activity of other human steroid receptors such as GR, AR, MR, and ER, whereas it had no observable effect on the activity of other members of the nuclear receptor superfamily (Tung *et al.,* 1993; McDonnell and Goldman, 1994; McDonnell *et al.,* 1994; Wen *et al.,* 1994; Kraus *et al.,* 1995). From a biological and pharmacological perspective, the most intriguing finding was that hPR-A functioned as a transdominant repressor of hER transcriptional activity in the presence of both agonists and antagonists (Vegeto *et*

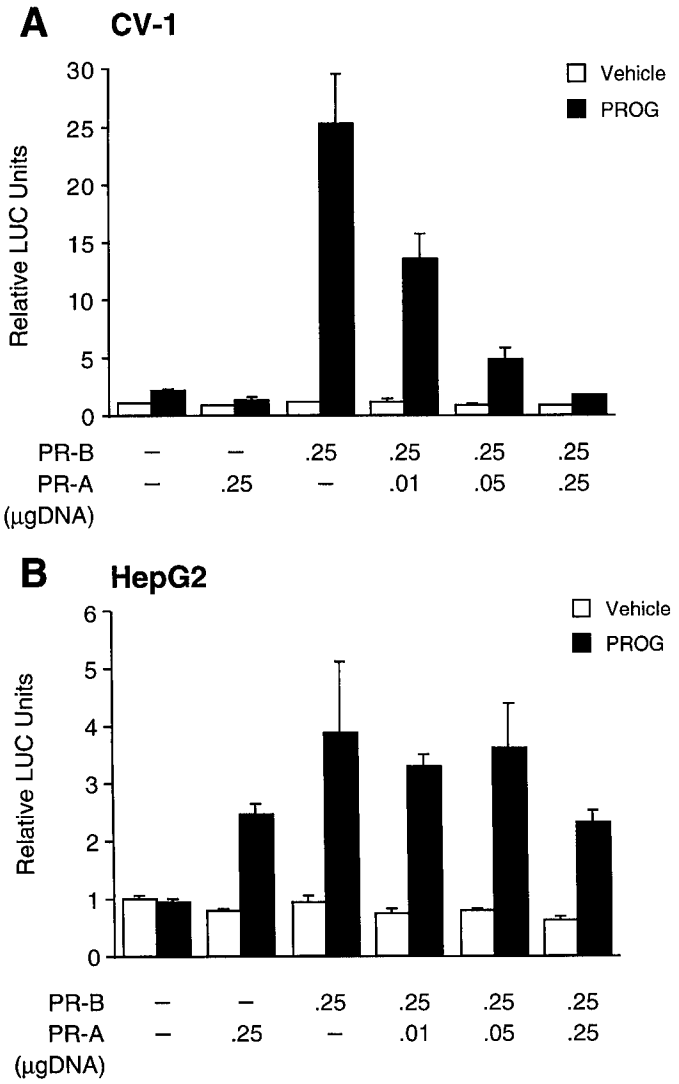


FIG 4. hPR-A functions as a transrepressor of hPR-B transcriptional activity. CV-1 (A) or HepG2 (B) cells were transiently transfected with either 0.25 μg phPR-B, phPR-A alone, or phPR-B in the presence of increasing concentrations of phPR-A together with 5 μg PRE2tk-LUC reporter and 5 μg pCH110 as an internal control. Cells were treated with or without 10^{-7} M progesterone as indicated for 24 hours and assayed for β-galactosidase and luciferase activity (luciferase activity was normalized to β-galactosidase activity). The relative luciferase activity (LUC activity) is calculated by dividing the normalized luciferase value at a given point by that obtained in the absence of transfected receptor or ligand. The data shown are representative of several experiments and indicate the mean ± average deviation from the mean triplicate estimations.

al., 1993; McDonnell and Goldman, 1994; Wen *et al.,* 1994; Giangrande *et al.,* 1997). This activity is illustrated in Figure 5. Specifically, it was shown that, in the presence of hPR-A, both R5020, a synthetic progestin, and RU486 were capable of suppressing hER transcriptional activity. Interestingly, RU486-bound hPR-A was a better transrepressor of ER transcriptional activity. hPR-B, importantly, does not repress hER activity under these conditions (McDonnell and Goldman, 1994; Giangrande *et al.,* 1997). Evidence in support of the biological relevance of hPR-A's function comes from studies done in rats, where it was shown that RU486 administration led to the downregulation of estradiol-mediated transcription without affecting ER expression levels (Kraus and Katzenellenbogen, 1993). We believe, therefore, that the transdominant activity of hPR-A may be responsible for regulating the cross-talk between the estrogen and progesterone signaling pathways that occurs in the reproductive tract. The data have also caused us to question whether the observed cellular responses to progestins and antiprogestins are due to their ability to modulate hPR-B or hER transcriptional activity or if, in fact, both activities are required. Answers to these important questions will emerge in the near future, as mice bearing specific hPR-A or hPR-B deletions become available.

III. An Inhibitory Domain Within hPR Is Responsible for hPR-A-mediated Repression of Estrogen Receptor Transcriptional Activity

Although several systems have been defined in which two forms of a transcription factor have different and opposing activities, the mechanisms by which the negative regulatory activity occurs vary from system to system. Some inhibitory proteins work by sequestering a limiting transcription co-factor, a process known as *squelching,* whereas others function by inhibiting the interaction of the positive transcription factor with target DNA. In the early stages of our work, we showed that hPR-A inhibited not only hPR-B transcriptional activity but also the transcriptional activities of ER and of other steroid hormone receptors. Thus, it was unlikely that heterodimerization or direct competition for a DNA-regulating event was possible. The remote possibility that these processes were involved was ruled out by experimentation (Vegeto *et al.,* 1993). Based on these findings, it was likely that some sort of transcriptional interference mechanism was operative. Furthermore, we concluded from these observations that hPR-B and hPR-A were very different transcription factors and are best considered as receptors that just happen to be regulated by the same hormone. Based on this premise, we undertook to define the specific domain(s) within hPR-A that were responsible for its unique transcriptional activity. A first step, we believed, was identifying factors that distinguished hPR-B, a transcriptional activator, from hPR-A, a transcriptional repressor.

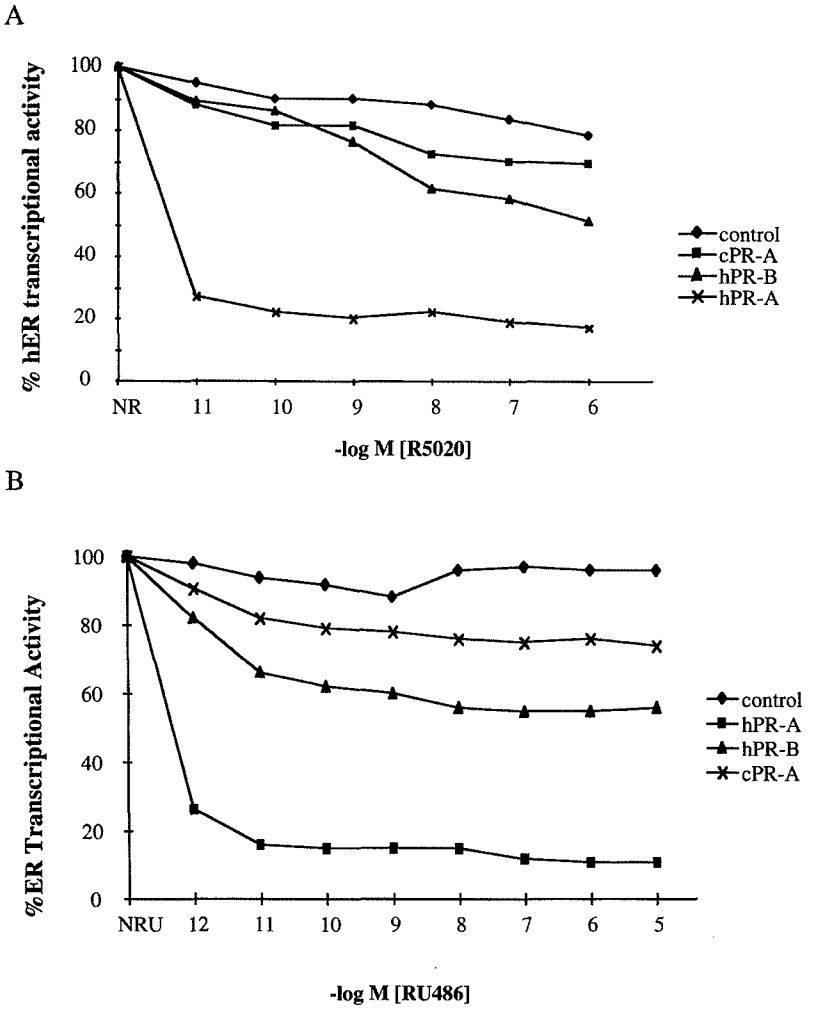


FIG 5. hPR-A, but not hPR-B or cPR-A, can function as a transrepressor of hER transcriptional activity. HeLa cells were transiently transfected with vectors expressing the human estrogen receptor alone or in combination with a vector expressing hPR-A (pBK-hPR-A), hPR-B (pBK-hPR-B), or cPR-A (pBK-cPR-A), respectively. The transcriptional activity of these constructs was measured following the addition of 10^{-7} M 17-β-estradiol alone or in combination with increasing concentrations of R5020 (A), a progesterone synthetic analog, or RU486 (B), an antiprogestin. In these experiments, estrogen receptor transcriptional activity was assayed on a 3XERE-TATA-LUC reporter. The data are presented as % activation, where 100% represents a measure of 17-β-estradiol-dependent transactivation by hER in the presence of a control vector, pBK-Rev-TUP1, or in the presence of hPR-A, hPR-B, or cPR-A, respectively, all in the absence of added PR ligands. This value is independently calculated for each data point. The average coefficient of variation at each hormone concentration was < 10%. NR = no R5020; NRU = no RU486.

It has been postulated that hPR-B contains three specific activation functions (AFs 1–3), whereas hPR-A only has two. Specifically, AF-1, located within the amino terminus, and AF-2, in the carboxyl terminus, are contained within sequences that are common to both hPR-A and hPR-B. Interestingly, however, a third potential activation function, AF-3, has been identified within the first N-terminal 164 amino acids of hPR-B (BUS; B upstream sequence), a region that is absent in hPR-A (Sartorius *et al.,* 1994). Thus, it is possible that hPR-A is transcriptionally inactive because it lacks a third activation domain. However, since AF-3 only functions in an autonomous manner when it is fused to an intact PR-DBD and will not activate transcription when fused to a heterologous DBD (Meyer *et al.,* 1992; Sartorius *et al.,* 1994), it's more likely that BUS, instead of functioning as a classical AF, contains sequences necessary for maximal AF-1 and AF-2 transcriptional activity (Giangrande *et al.,* 1997). Thus, BUS may contribute to hPR-B transcriptional activity in a direct manner by enhancing the activity of AF-1 or AF-2, or it may work as an antirepressive sequence by suppressing the activity of a repressor domain contained within sequences common to hPR-A and hPR-B (Kastner *et al.,* 1990). Evidence in support of this latter hypothesis came from studies where we found that the A and B isoforms of the human and chicken progesterone receptors were different both functionally and in terms of primary sequence (Figure 3). Specifically, analysis of the transcriptional activity of the chicken PR isoforms, cPR-A and cPR-B, revealed that these two receptors, like hPR-A and hPR-B, have similar DNA- and ligand-binding properties; however, unlike their human counterparts, both chicken PR isoforms functioned as potent activators of progesterone-responsive genes in a context-independent manner (Gronemeyer *et al.,* 1987; Tora *et al.,* 1988; Conneely *et al.,* 1989; Giangrande *et al.,* 1997). Thus, cPR-A, although similar in sequence and structure to hPR-A, functioned as an activator and not an inhibitor of progesterone-responsive genes (Krust *et al.,* 1986; Conneely *et al.,* 1987; Misrahi *et al.,* 1987; Giangrande *et al.,* 1997). Subsequently, based on these results, we undertook a chicken/human domain-swapping approach, to see if a discrete inhibitory region could be mapped within hPR-A.

The most extensive differences in primary structure between the chicken and human PR-As are found within the N-terminal domains of these receptors (Giangrande *et al.,* 1997). Using this information, a series of chimeric proteins were created in which the least-conserved regions of chicken and human receptors were swapped. Subsequent analysis of the ability of these chimeras to modulate hER transcriptional activity allowed us to map a specific inhibitory region within hPR-A. Specifically, we demonstrated that the first 140 amino acids of hPR-A (aa 165–305) were necessary for its ability to function as a transcriptional inhibitor as well as a transrepressor of heterologous steroid receptor transcriptional activity (Giangrande *et al.,* 1997). Interestingly, deletion of the first N-terminal 140 amino acids from hPR-A resulted in a receptor mutant that is functionally

indistinguishable from hPR-B (Giangrande *et al.,* 1997). Furthermore, although this repressor region is transferable, it does not function as an autonomous transrepressor when fused to GAL4-DBD, suggesting the importance of other domains of the receptor for proper repressor activity. Recently, the Horwitz laboratory has shown that the hPR-A repressor domain inhibits AF-1 and AF-2 but not AF-3 (Hovland *et al.,* 1998). In addition, fusion of this repressor domain onto hER strongly represses transcription by this receptor (Hovland *et al.,* 1998). Cumulatively, these results support the hypothesis that hPR-A, like hPR-B, contains all the sequences necessary for proper transcriptional activation. However, in the absence of the B-specific 164 amino acids, the repressor function present within the hPR-A amino terminus prevents AF-1 and/or AF-2 from activating transcription. It follows, then, that the role of the B-specific sequences (BUS) is to override this inhibitory function and permit hPR-B to activate transcription (Giangrande *et al.,* 1997; Hovland *et al.,* 1998).

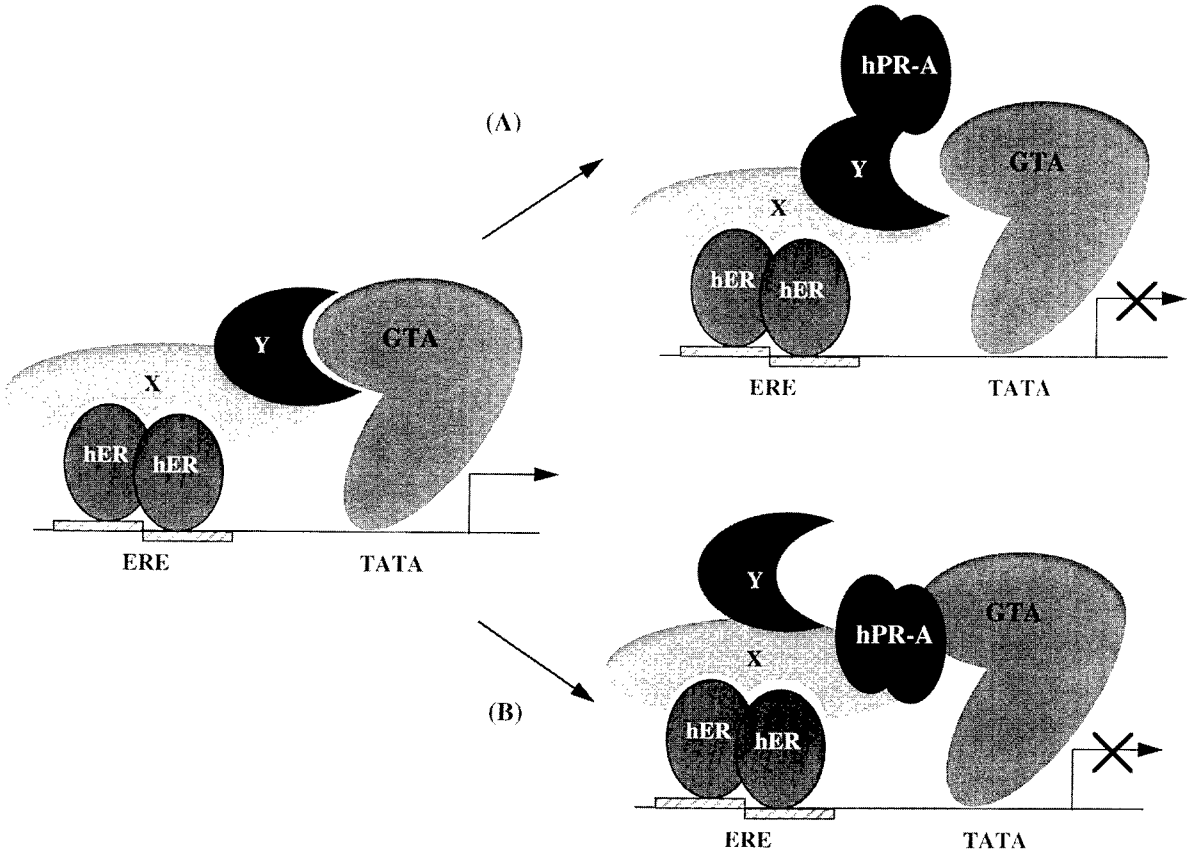
IV. Mechanism of hPR-A-mediated Transrepression of Steroid Hormone Receptors Transcriptional Activity

In addition to hPR-B, several other transcription factors have been identified that contain both activation and repression functions. Examples of such factors include the lymphoid-specific transcription factor, Oct-2a (Friedl and Matthias, 1995); members of the AP1 family of transcription factors c-Fos, c-Jun, and the related protein FosB (Baichwal and Tijan, 1990; Baichwal *et al.,* 1992; Brown *et al.,* 1995); and a member of the basic region-leucine zipper (bZIP)-containing family of transcription factors, ATF-2 (Li and Green, 1996). Specifically, repressor domains within these other proteins were identified by creating deletions that enhanced their overall transcriptional activity (Baichwal and Tijan, 1990; Baichwal *et al.,* 1992; Dubendorff *et al.,* 1992; Brown *et al.,* 1995; Fried and Matthias, 1995; Li and Green, 1996). Interestingly, the inhibitory domain (ID1) present at the amino terminus of c-Fos has been shown to specifically silence an N-terminal activation function containing HOB1 motifs (Brown *et al.,* 1995). Similarly, the c-Fos-related protein, FosB, also contains an inhibitor domain (inhibitor motif, IM1) that, when mutated, enhances the ability of c-Fos to activate an AP1-bearing promoter. Interestingly, overexpression of c-Fos ID1 alone alleviates the inhibitory effect of ID1 present within c-Fos (Brown *et al.,* 1995). This de-repression suggests that ID1 binds to and sequesters a limiting protein involved in repression of c-Fos activity. Whether the mechanism of hPR-A-mediated transrepression of steroid hormone receptor activity involves binding to a co-repressor protein is still unknown. However, our unpublished data suggest that the hPR-A deletion mutant lacking the N-terminal repressor domain forms a weaker interaction with the co-repressor SMRT than does full-length hPR-A.

Based on the studies outlined above, we considered that hPR-A is repressing transcription of other steroid receptors by a nonclassical mechanism known as *transcriptional interference* (Figure 6) (Meyer *et al.,* 1989,1992). This form of transcriptional antagonism has been documented for various pairwise combinations of steroid receptors: ER, GR, PR, and also between TR or RAR and GR or PR (Meyer *et al.,* 1989; Barettino *et al.,* 1994; Yen *et al.,* 1995; Zhang *et al.,* 1996). Transcriptional interference results from the disruption of a distal step in the signaling pathway of a transcriptional activator (i.e., hER) necessary for its proper transcriptional activity. Transcriptional interference can be either direct or indirect. Direct or competitive interference occurs due to the competition for a common limiting factor required by a transcriptional activator for proper activity. For example, if the direct mechanism was operative, the requirement of hER for this limiting factor would be determined by cell and promoter context such that hPR-A would not inhibit all estrogen-induced target genes. Indirect or noncompetitive inhibition, on the other hand, could occur as a consequence of competition for distinct targets or different sites on a common target protein.

Our early studies addressed the possibility that hPR-A was titrating a factor required for steroid receptor transcriptional activation (Wen *et al.,* 1994). Interestingly, however, these studies revealed that the inhibitory activity of hPR-A was independent of the concentration of activating receptor but was dependent on the absolute expression level of hPR-A within the cell. This suggested to us that squelching, a competitive phenomenon, was not the mechanism of hPR-A-mediated transrepression but rather that hPR-A was functioning in an indirect, noncompetitive manner to repress SHR action (Wen *et al.,* 1994). In light of these important data, we now believe that an indirect form of transcriptional interference is the most likely mechanism for hPR-A transdominant repression of steroid receptor action (Vegeto *et al.,* 1993; Wen *et al.,* 1994; McDonnell and Goldman, 1994). Specifically, we propose that hER can recruit one (X) or more co-activators (X and Y), which act as bridging factors to allow hER to contact the general transcription machinery (GTA) and activate transcription (Figure 6). Therefore, hPR-A can prevent hER from contacting the GTA in either of two ways. One

FIG 6. Mechanism of hPR-A transdominant inhibition of hER function. Based on studies reviewed within, we propose that hPR-A represses transcription of other steroid receptors by a nonclassical mechanism known as transcriptional interference. In this model, hER can recruit one (X) or more co-activators (X and Y) that allow ER to contact the general transcription machinery (GTA) and activate transcription of target genes. hPR-A can prevent ER from contacting the GTA in either of two ways: (A) hPR-A can interact with different sites on a common co-activator (X) that binds to ER, or (B) hPR-A can bind a co-activator (Y) that does not interact directly with ER. In both scenarios, hPR-A sterically hinders hER from contacting the GTA. Given this model, it is conceivable that hPR-A could further hinder hER from contacting the GTA by recruiting a co-repressor to the ER-co-activator complex.


(A)
hPR-A
Y
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possibility is that hPR-A and hER interact with different sites on a common co-activator, denoted "X." The interaction of hPR-A with X prevents the association of hER with the general transcription apparatus, thus inhibiting hER-mediated transcription (Figure 6A). A second possibility is that hPR-A binds to co-activator Y and, likewise, prevents hER from contacting the GTA and activating transcription. In this scenario, hPR-A and hER interact with distinct proteins within the co-activator complex (Figure 6B).

An important component of these models is that hPR-A is able to interfere with the transcriptional machinery in a specific manner, in the absence of being able to interact directly with a progesterone-responsive element. Although, within the confines of classical PR action, this seems hard to understand, the recent "receptosome" model proposed by O'Malley and co-workers may be relevant to hPR-A action (McKenna *et al.,* 1998). Specifically, they propose that ligand-activated receptors permit the formation of a large, oligomeric complex containing the receptor and a large number of activator proteins, the transcriptional activity of which is influenced by cell and promoter context. Thus, hPR-A could operate by disordering this complex in some manner, or it could bring an inhibitory activity to the complex. If this model is shown to be correct, then we would postulate that, in the case of hER and hPR-A, both receptors, in the presence of ligand, can join the "receptosome" complex. ER permits the complex to bind to EREs, while the presence of hPR-A prevents this from being a productive interaction. Thus, transcriptional interference of hER activity by hPR-A can result from a combination of both steric hindrance (Figure 6) and recruitment by hPR-A of a co-repressor to the hER co-activator complex. The identification of such hER and hPR-A interactors is crucial to resolve these issues. The idea of a "receptosome" is intriguing. Even though its existence remains to be proven biochemically, it is useful to think of such a unit existing functionally in order to understand hPR-A function. Though these are early days in this particular field of research, there is an accumulating amount of evidence that the "receptosome" contains several receptor-associated proteins that can function as either positive or negative regulators of steroid hormone action. Thus, this next section will review what is known about transcriptional co-activators and co-repressors that could permit the link between hPR-A and other nuclear receptors.

V. PR and ER Co-activators/Integrators

Conceptually, the target of transcriptional interference may be either a basal transcription factor or a co-activator. In transient transfection experiments, however, hPR-A does not alter basal transcription, indicating that transrepression by hPR-A is unlikely to be due to inhibition of the activity of a basal transcription factor (Giangrande *et al.,* 1997). It follows, then, that the point of convergence of hPR-A and hER is likely to be a transcriptional co-activator(s) that is required

for ER transcriptional activity (McDonnell and Goldman, 1994; Wen *et al.,* 1994; McDonnell *et al.,* 1994; Giangrande *et al.* 1997). Interestingly, most of the known ER co-activators require a functional AF-2 domain within ER to manifest activity. However, we have shown that an hPR-A mutant lacking a functional AF-2 was an effective inhibitor of ER transcriptional activity (Wen *et al.,* 1994). This suggests that, if ER and hPR-A are interacting with the same co-activator, they do not utilize the same contact sites. A brief summary of the potential co-activators involved in this process will serve to evaluate the potential target(s) for hER and hPR-A convergence.

Among the many nuclear receptor co-activators identified in recent years, only four have been shown to specifically modulate both PR and ER transcriptional activity and, as such, may be targets for ER/PR cross-reactivity. These are SRC-1 (Oñate *et al.,* 1995), the SRC-1-related proteins, GRIP-1/TIF-2 (Hong *et al.,* 1996; Voegel *et al.,* 1996), CBP (Chakravarti *et al.,* 1996; Kamei *et al.,* 1996; Smith *et al.,* 1996), and p/CIP (Torchia *et al.,* 1997). Although it is unknown whether any of these co-activators is involved in hPR-A-mediated transrepression of hER activity, recent evidence suggests that SRC-1 and the p300/CBP-associated factor (pCAF) interact directly with the amino-terminal sequences of PR, albeit less efficiently than with AF-2, and mediate activation through the amino terminus of both PR and ER (Oñate *et al.,* 1998; Jenster *et al.,* 1997; Smith *et al.,* 1997). SRC-1 was isolated following a yeast two-hybrid screen of a human cDNA library using PR-HBD as bait (Oñate *et al.,* 1995). This factor interacts with the receptor only in the presence of agonist and similarly enhances hPR-B transcriptional activity in the presence of agonist R5020 but not the antagonist RU486. Furthermore, a dominant negative of SRC-1, SRC-1(0.8), which contains the receptor-interacting domains but not the N-terminal activation domains, suppresses PR transcriptional activity both *in vivo* and *in vitro* (Jenster *et al.,* 1997). SRC-1 also enhances *in vivo* transcription by GR, ER, TR, and RXR; its overexpression reverses ER-mediated squelching of hPR-B transcriptional activity (Oñate *et al.,* 1995). In addition to interacting with a variety of nuclear receptors, SRC-1 also interacts with TBP and TFIIB and thus may be functioning as a bridge between nuclear receptors and the general transcription machinery (Takeshita *et al.,* 1996). Although already complex, the identification of SRC-1-related co-activators, such as TIF-2, whose activities are similar though distinct from SRC-1, suggests that PR and other nuclear receptors may utilize several different mechanisms to modulate target gene transcription. TIF-2 was identified as a 160 kDa protein that interacts in a ligand-dependent manner with ER-HBD and RAR (Voegel *et al.,* 1996). In addition, TIF-2 was also shown to interact in a ligand-dependent manner with the AF-2 domain of PR (Voegel *et al.,* 1996). Its mouse homologue, the glucocorticoid receptor-interacting protein (GRIP-1) was independently identified by a yeast two-hybrid screen of a mouse embryo cDNA library (Hong *et al.,* 1996). Recently, SRC-1 was shown to possess intrinsic

histone acetylase activity as well as to interact with a histone acetyl transferase (HAT) protein, pCAF (CBP-associated factor) (Spencer *et al.,* 1997). It was suggested that SRC-1- and pCAF-mediated acetylation of histones bound to specific promoters is dependent on ligand binding to steroid receptors. Thus, one could envision a mechanism by which the AFs of steroid receptors and their co-activators enhance formation of a stable preinitiation complex that increases transcription of specific genes from repressed chromatin templates (Spencer *et al.,* 1997; Shibata *et al.,* 1997) and that hPR-A, if introduced into the complex, would prevent ER-SRC-1 interactions from being productive.

In addition to the SRC-1 family of co-activators, it has been shown recently that the amino terminus of the CREB-binding protein, CBP, interacts with a subset of nuclear receptors, including ER and PR via their LBDs (Kamei *et al.,* 1996; Hanstein *et al.,* 1996; Chakravarti *et al.,* 1996). In addition, the carboxyl terminus of the protein has been shown to interact with many of the nuclear receptor co-activators such as SRC-1, GRIP-1/TIF2, and the p300/CBP/-integrator-associated protein, p/CIP (Hanstein *et al.,* 1997; Smith *et al.,* 1996; Yao *et al.,* 1996; Kamei *et al.,* 1996; Torchia *et al.,* 1997). Thus, the ternary complex, steroid receptor-co-activator-CBP, appears to be essential for steroid receptor-mediated transcriptional activity (Kamei *et al.,* 1996; Chakravarti *et al.,* 1996). Interestingly, Smith *et al.* (1996) have shown that co-expression of CBP and SRC-1 stimulates ER and PR-B transcription in a synergistic manner and that CBP alone is able to partially reverse the ability of active ER to squelch PR-B-dependent transcription. This suggests that different steroid hormone receptors can antagonize one another in a ligand-dependent fashion by binding and sequestering these limiting co-activator proteins (Zhang *et al.,* 1996). It is possible that hPR-A might inhibit the transcriptional activity of heterologous steroid receptors underlying a similar mechanism. One could envision a scenario in which hPR-A binds SRC-1 and/or CBP with greater affinity than either hPR-B or hER and thus sequesters this co-activator complex and suppresses steroid hormone receptor activity. It is possible that the unmasked repressor domain of hPR-A (Giangrande *et al.,* 1997) allows this isoform of human PR to recognize different sites on these co-activators than those recognized by hPR-B. This, in turn, might increase the affinity of hPR-A for these factors and inhibit binding of other steroid hormone receptors to these proteins. Interestingly, recent studies (Torchia *et al.,* 1997; Heery *et al.,* 1997) have mapped two distinct nuclear receptor-interacting domains on CBP. These domains contain a short sequence motif LXXLL (where L is leucine and X is any amino acid) present in many steroid hormone receptor co-activators (Torchia *et al.,* 1997; Heery *et al.,* 1997). Thus, two different steroid receptors (i.e., hPR-A and hER) could interact simultaneously with CBP and the binding of one of the receptors (i.e., hPR-A) might antagonize the binding and/or the proper function of the other receptor.

In addition to the well-characterized proteins, the co-activator p/CIP could also be important in the communication between hER and hPR-A. p/CIP was identified in a screen for CBP- and ER-interacting proteins (Torchia *et al.,* 1997). Even though overexpression of this co-activator increases steroid hormone receptor transcriptional activity only marginally, microinjection of antibodies specific for p/CIP prevents transcription of ER, PR, and other nuclear receptors. This repression can only be overcome by overexpression of both p/CIP and CBP, suggesting that CBP-p/CIP complex is necessary for steroid receptor-mediated transcription (Torchia *et al.,* 1997).

While the co-activators identified so far have been shown to interact preferentially with hydrophobic residues in helix 12 of nuclear receptors and potentiate AF-2 function, no AF-1-specific co-activators have been found (Barettino *et al.,* 1994; Danielian *et al.,* 1992; Durand *et al.,* 1994; Renaud *et al.,* 1995; Saatcioglu *et al.,* 1993). This is not surprising, since the *in vivo* and *in vitro* screens used to isolate these proteins were done primarily using the LBDs of nuclear receptors as bait. It is likely, then, that additional co-factors exist that bind preferentially to the N-terminus of nuclear receptors or require the intact receptor context in order to bind and transactivate nuclear receptor transcriptional activity. Therefore, although these AF-2 co-activators are important for proper transcriptional activity of nuclear receptors, it is clear that they may not be the only factors involved in the intrinsic biological activities displayed by hPR-A. It is not known if any of the above co-activators are responsible for hPR-A-mediated repression of hER transcriptional activity. However, the ability to characterize specific domains within ER and hPR-A responsible for cross-talk between the receptors, and the success of others in identifying co-activators involved in transcriptional activation by many steroid receptors, suggest that the isolation and identification of factors required for hPR-A repressive activity will likewise be successful.

VI. The Involvement of Nuclear Receptor Co-repressors in PR/ER Cross-talk

As mentioned earlier, we must not rule out the possibility that the differential effects of hPR-A are due in part to its association with co-repressor proteins or silencing mediators such as SMRT, NCoR, and SUN-CoR (Chen and Evans, 1995; Hörlein *et al.,* 1995; Zamir *et al.,* 1997). Indeed, negative transcriptional regulation by the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) is mediated in part by their association with these silencing mediators. To this effect, our unpublished data suggest that hPR-A has greater affinity for SMRT than does hPR-B in the presence of antagonist, RU486, in a mammalian two-hybrid assay. This suggests that the amino acid sequences in the amino terminus of hPR-B are important regulators of co-repressor interactions. Furthermore, differential co-repressor association may explain, in part, why hPR-B is an efficient

transcriptional activator of most progesterone-responsive genes and why hPR-A, which has greater affinity for co-repressors, acts as a repressor of most progesterone-responsive genes. It is also possible that hPR-A transrepresses steroid receptor transcriptional activity by bringing to the ternary complex (steroid hormone receptor-co-activator-transcription factor) a strong repressor protein such as SMRT. One could then envision a scenario in which the repressive function of the silencing mediator, SMRT, would be dominant over the activator function of the ternary transcription complex.

Examples of nuclear receptor co-repressors are SSN6 (McDonnell *et al.,* 1992), SMRT (Chen and Evans, 1995), NCoR (Hörlein *et al.,* 1995), and SUN-NCoR (Zamir *et al.,* 1997). SSN6 was shown to repress ER and hPR-B transcriptional activity in yeast (McDonnell *et al.,* 1992). Similarly, both SMRT and NCoR have been shown to interact with steroid hormone receptors and, in particular, with both isoforms of the hPR (Wagner *et al.,* 1998).

The mouse nuclear receptor co-repressor, NCoR, was isolated in a yeast two-hybrid screen using unliganded TRβ as bait (Hörlein *et al.,* 1995). NCoR is a 270 kDa protein that has three transferable repressor domains located at its amino terminus and multiple receptor interaction domains at its carboxyl terminus. Another co-repressor, SMRT (silencing mediator of retinoid and thyroid hormone receptors), was isolated in a similar two-hybrid screen using an unliganded hRXRα-HBD fusion protein as bait (Chen and Evans, 1995). Like NCoR, SMRT is a widely expressed nuclear protein with a strong N-terminal repressor domain and a C-terminal receptor interaction domain. SMRT and NCoR represent a new class of transcriptional mediators for nuclear receptors that have been shown to actively silence basal transcription (Perlmann and Vennström, 1995). The recently identified co-repressor, SUN-CoR (small unique nuclear receptor co-repressor), shares no homology with the previously described nuclear hormone co-repressors, NCoR or SMRT (Zamir *et al.,* 1997). SUN-CoR has been shown to potentiate transcriptional repression by thyroid hormone receptor and RevErb *in vivo* and to interact directly with TR as well as with RevErb *in vitro;* however, it is not known whether this co-repressor is capable of associating with either ER or PR. Interestingly, SUN-CoR has also been shown to associate with NCoR and SMRT *in vitro* and to bind to endogenous NCoR in cells, suggesting that a complex of co-repressors may be involved in transcriptional transrepression by unliganded and orphan nuclear hormone receptors (Zamir *et al.,* 1997).

The nuclear receptor co-repressor proteins identified to date have been shown to share a basic structure composed of multiple N-terminal repressor domains and one or more C-terminal receptor interaction domains (Hörlein *et al.,* 1995; Seol *et al.,* 1996). Like co-activators, co-repressors do not bind directly to DNA and must associate with DNA-binding proteins in order to repress gene transcription. Furthermore, the transcriptional silencers, NCoR and SMRT, have been shown to exist in a complex with mSin3, a yeast transcriptional repressor, and the histone

deacetylase HD-1 (also known as HDAC1). Given these observations, it has been postulated that co-repressors mediate gene repression by acting as bridging factors between the receptor and HDs, thus recruiting HDs to the receptor-DNA complex (Alland *et al.,* 1997; Heinzel *et al.,* 1997). Unlike co-activators, these transcriptional silencers have also been shown to modulate basal transcription as well as transcription of a wide range of nuclear receptors (Hörlein *et al.,* 1995; Chen and Evans, 1995; Seol *et al.,* 1996; Wagner *et al.,* 1998).

VII. Final Comments

The classical models of PR action suggest that all the biological actions of progesterone are manifest through a single receptor that is biochemically identical in all cells. From this simple model, it follows that the pharmacology of PR agonists and antagonists is likewise simple. Specifically, it was inferred that the function of an agonist is to convert PR from a transcriptionally inactive form to one that interacts with specific DNA response elements within target genes and positively or negatively regulates its transcription. Thus, the agonist functions as a switch. When corrected for affinity, therefore, all agonists were believed to be quantitatively the same. Antagonists, on the other hand, were predicted to function in a simple, competitive manner, blocking agonist access to the receptor. Over the past 10 years, however, it has become increasingly clear that the pharmacology of progestins and antiprogestins is much more complex. Specifically, it has now been demonstrated that different ligands have different effects on PR structure and that cells can distinguish between different PR-ligand complexes. Compounding this issue of complexity even further was the identification of a second functional progesterone receptor, hPR-A, whose activity was dissimilar to that exhibited by its larger hPR-B counterpart. One of the most interesting and potentially important activities manifest by hPR-A is its ability to modulate the transcriptional activity of nuclear receptors other than PR. Thus, some of the biological responses of a cell to progesterone agonists and antagonists relate to their ability, through hPR-A, to regulate estrogen, androgen, mineralocorticoid, and glucocorticoid receptor transcriptional activity. Taken all together, it is apparent that PR pharmacology is much more complex than was originally thought and it is likely to get even more complex as the precise mechanism of action of hPR-A is determined. Although daunting from a mechanistic point of view, this complexity provides opportunities for the development of PR modulators that manifest their biological activity in a cell- or promoter context-specific manner, an activity that will be facilitated by ongoing genetic and pharmacological studies of the PR signaling pathway.

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DISCUSSION

Amita Sehgal: "Transcription-independent mechanisms" in the case of estrogen receptors are not necessarily transcription independent. Couldn't estrogen be activating transcription through the other signaling pathways?

Donald McDonnell: I certainly agree. I have used the term "transcription independent" to describe actions of steroid hormones that occur within a few minutes of the addition of the specific agonist. Thus, I mean to imply "independent" of the transcriptional events mediated by estrogen receptor (ER) through its cognate response elements. I do not mean to imply that rapid responses of cells to steroid hormones, like activation of MAPK, do not impact transcription within a target cell.

Amita Sehgal: Are estrogen targets in bone known and does tamoxifen activate those targets?

Donald McDonnell: As yet, there is very little known regarding the mechanism by which ER functions in bone. One attractive hypothesis is that ER, in the presence of agonists or antagonists, can inhibit the transcriptional activity of the transcription factor C/EBP and thus repress interleukin-6 synthesis. However, it's likely to be more complex.

Evan Simpson: Do inhibitors of MAPK block the proliferation response of MCF-7 cells to estradiol? Do estrogens activate the ERK pathways, for example, P38/HOG?

Donald McDonnell: In fact, they appear to do so. A presentation at the recent Endocrine Society annual meeting by the Santen laboratory showed, in an elegant series of experiments, that the mitogenic action of estradiol in MCF-7 cells could be blocked by inhibitors of the MAPK signaling pathway. We have looked at other signaling pathways but as yet have no evidence to suggest that estradiol can activate pathways other than those that impinge on p42/44 MAPK.

Martha Gillette: Please elaborate on the likelihood that EGF leading to MAPK activation leads to transcriptional activation at non-ER response elements and that changes in Ca^{2+} may lead to transcriptional activation at other sites (Ca^{2+}/Ca response elements). The potential for activation at these sites together or estrogen receptor response elements can lead to a richness and complexity in transcriptional change.

Donald McDonnell: In our studies, we have been able to show that MAPK activation by estradiol is a rapid event that appears to involve a similarly rapid release of Ca^{2+} from intracellular stores. But, as you rightly point out, an event such as estrogen-induced calcium release is likely to do more within the cell than just lead to MAPK activation. We are in the process of trying to see if other calcium-sensitive systems are affected by the magnitude of the calcium changes that occur upon estrogen treatment.

William W. Chin: Your data and those dealing with SERMs suggest that the tissue context is a critical variable in evaluating estrogen and analog action. However, in these studies, you have focused mainly on a single consensus ERE. Do these conclusions hold for different EREs? In this light, what

do we mean when we say "tissue-specific" context? Perhaps tissue-specific genes have slightly modified versions of EREs, etc. to explain those differential effects. Recently, Lannigan's group has shown direct interaction of ER with p90 rsk(?) kinase. In light of your interesting MAP kinase data suggesting a nongenomic effect of estrogen, what data are available indicating that the estrogen receptor interacts with factors other than COAs and CoRs? Regarding your data on cAMP and PR interactions, have you examined the roles of phosphorylated PR or other factors such as CoAs, CoRs, and CBP in this phenomenon?

Donald McDonnell: A search of the literature indicates that ER has been shown to interact with proteins other than those that would be classified as classical co-activators or co-repressors. However, to date, a clear idea as to the relevance of these latter interactions has not emerged. Nancy Weigel's group in Houston has been unable to show a significant change in PR phosphorylation upon cAMP treatments. Thus, we believe that cAMP interrupts PR-co-repressor interactions, either by changing the phosphorylation state of a co-repressor and promoting displacement from PR or by changing the phosphorylation state of a co-activator and enhancing its ability to interact with PR. The latter reaction, we suggest, would lead to a subsequent displacement of the co-repressor.

Susan Davis: Were the levels of 17β-estradiol required to achieve MAP kinase intracellular calcium responses in MCF-7 cells physiological? Since exogenous estrogens/tamoxifen/raloxifen all result in equal incidence of deep venous thrombosis and pulmonary embolism, is anything known about the mechanism involved? (Knowing that leiden mutation is a complementary risk factor.)

Donald McDonnell: Under the condition of our assay, we were able to see alterations in MAPK activity at physiological concentrations of estradiol. I do not know of any mechanistic data that could explain the effect of ER ligands on the incidence of deep venous thrombosis. This, of course, is a very important issue and drugs that function as estrogens but that do not display this activity are clearly needed. In my presentation, I focused on the role of conformation of receptor and how this influences the ability of the estrogen and progesterone receptors to interact with transcription co-activators and co-repressors. However, we have performed additional studies that have examined the effect of the DNA response element on the pharmacology of ER ligands. As you might expect, the sequence of the response element and the promoter context in which it was studied had a profound effect on ER response to various ligands. Specifically, in the case of ER, we found some DNA elements on which estradiol-activated ER alone could activate transcription, whereas others were identified that were much more promiscuous. Interestingly, some recent work from the Narduli laboratory has indicated that the structure of ER, when bound to different response elements, is not identical.

Appendix #5

10 Functional and Pharmacological Analysis of the A and B Forms of the Human Progesterone Receptor

P. Giangrande, G. Pollio, and D.P. McDonnell

10.1 Introduction

The steroid hormone progesterone is a key regulator of the processes involved in the development and maintenance of reproductive function (Clark and Peck 1979; Clarke and Sutherland 1990). In addition, however, the efficacy of antiprogestins as treatments for brain meningiomas, breast cancer, uterine fibroids, and endometriosis have implicated progesterone in the pathology of these diseases (Poisson et al. 1983;

Colletta et al. 1991; Kettel et al. 1991; Horwitz 1992; Lundgren 1992; Brandon et al. 1993; Carroll et al. 1993). The mechanism by which progesterone manifests biological activity in target tissues is similar to that of other members of the steroid hormone receptor superfamily (McDonnell 1995). In mammals progesterone is transported in the blood throughout the body and is capable of diffusing across all cell membranes; however, it exerts biological activity only in those cells which express a specific high affinity nuclear progesterone receptor (PR).

In the absence of progesterone, PR resides in an inactive state within the nuclei of target cells associated with a large multi-component heat-shock protein complex (McDonnell 1995). Upon hormone binding, however, the receptor undergoes a conformational change, an event which leads to the dissociation of the receptor from the inhibitory heat-shock protein complex and the subsequent formation of stable homodimers (Vegeto et al. 1992). In this form the receptor is capable of interacting with specific high affinity DNA sequences (progesterone response elements; PREs) located within the regulatory regions of target gene promoters and is capable of positively or negatively regulating target gene transcription. Interestingly, this latter step in the signal transduction pathway appears to be the most complex and may not occur in the same manner in all cells and on all promoters. In some instances, it is now considered that the receptor can directly contact components of the general transcriptional machinery and that, in doing so, it can stabilize the transcription initiation complex (Jacq et al. 1994; Mengus et al. 1995; Schwerk et al. 1995). However, in addition to this mechanism, it is clear that the activated receptor can interact with the general transcription machinery in an indirect manner, through any of a number of intermediary cofactors (Halachmi et al. 1994; Cavaillès et al. 1995; Le Douarin et al. 1995; Oñate et al. 1995; Hanstein et al. 1996; Hong et al. 1996; Voegel et al. 1996). Of course these different pathways are not mutually exclusive but are differentially utilized in different cells. Overlaying this tremendous complexity is the fact that the PR, in most mammalian species, exists within target cells in either of two forms, PR-A or PR-B (Lessey et al. 1983). This complexity, however, provides an explanation for the cell-selective activities which are manifest by different PR ligands, and suggests further that it may be possible to develop pharmaceuticals that function as progestins or antiprogestins in a tissue-selective manner. This chapter specifically focuses on the role of the A and B forms of the PR in PR pharmacology and considers their utility as drug targets.

10.2 Two Different Forms of the Human Progesterone Receptor Exist in Target Cells

In humans, the two PR isoforms A and B arise from unique mRNA transcripts produced from a single gene by alternate transcription initiation (Kastner et al. 1990; Gronemeyer et al. 1991). Interestingly, under most circumstances hPR-A and hPR-B are co-expressed in target cells in approximately equimolar amounts (Lessey et al. 1983). Thus, as a consequence of the process of dimerization, the activated receptor can exist in the cell in either of three states, A:A, A:B, and B:B (DeMarzo et al. 1991, 1992). Because, as will be discussed in Sect. 10.3, the A and B forms of PR are not functionally equivalent, it is implied that the three dimeric states are also not functionally identical. Thus, the relative expression of the two isoforms is likely to be important in regulating cellular responsiveness to progestins and antiprogestins.

The initial characterization of the A and B forms of hPR indicated that they had equivalent affinities for target DNA and exhibited indistinguishable ligand-binding affinities and specificities (Lessey et al. 1983; Christensen et al. 1991). It was not apparent from these results, however, whether or not these PR isoforms were functionally different. In fact, it was suggested by some that the smaller A form of PR was derived in an artifactual manner from the B form as a result of proteolysis. However, the ontogeny of these receptors has now been elucidated for both chicken and human receptors. In the chicken, both receptors are derived from the same mRNA by alternate initiation of translation (Conneely et al. 1987). In humans, however, it appears as if both isoforms are derived from unique mRNAs which are produced from different promoters within the same gene (Kastner et al. 1990). These results, indicating that the cell has evolved complex regulatory mechanisms to regulate PR-A and PR-B expression, strongly suggested that both forms were important for PR action and were unlikely to be functionally redundant.

10.3 The Human Progesterone A and B Forms Manifest Distinct Activities Within Target Cells

Although the existence of two forms of PR had been reported in several species, it was not until their respective cDNAs were cloned and incorporated into reconstituted transcription systems that the significance of this finding was appreciated. The initial studies examined the transcriptional activity of the chicken PR-A and PR-B and determined that whereas both isoforms of cPR were capable of activating transcription, they each demonstrated a unique promoter specificity (Gronemeyer et al. 1987; Tora et al. 1988; Dobson et al. 1989). Several years later a similar analysis was performed using the cloned hPR-A and hPR-B

Fig. 1a–c. Differential transcriptional activities of human progesterone receptor-B (*hPR-B*), human progesterone receptor-A (*hPR-A*), chicken progesterone receptor-A (*cPR-A*). **a** Sequence similarities among PR isoforms. The DNA sequences of the human and the chicken isoforms of the PR were obtained from GenBank. Regions of amino acid similarities between hPR-A and cPR-A were determined using the DNA Strider and LALNVIEW programs. The regions of least homology are located upstream of the unique PmlI restriction site present in both receptors (55% similarity, 30% identity). Regions of high homology are found downstream of the PmlI restriction site (90% similarity, >72% identity). The amino acid sequence of the B isoform of the hPR is also shown. *HBD*, hormone-binding domain; *AF-1–3*, activation function-1–3; *DBD*, DNA-binding domain. **b** HeLa cells or HepG2 cells (**c**) were transiently transfected with increasing concentrations of vectors expressing hPR-B, hPR-A, or cPR-A ranging from 1 to 5 X, where X represents the respective concentration for each receptor corrected for molarity (where X = 0.131 µg for hPR-B, 0.12 µg for hPR-A, and 0.115 µg for cPR-A). The transcriptional activity was measured 24 h after the addition of 10^{-7} M R5020. In these experiments, PR transcriptional activity was assayed on a PRE2-TK-LUC promoter (1.5 µg). Transfections were normalized for efficiency using 0.05 µg of an internal β-galactosidase control plasmid (pBKC-βgal). Luciferase activity (*Luc. activity*) was normalized to β-galactosidase activity. The total concentration of cytomegalovirus (CMV) promoter was kept constant throughout the experiment by including the appropriate amount of a CMV-based control plasmid (pBK-Rev-TUP1). The total amount of DNA per triplicate was 3.0 µg. Each data point represents the average of triplicate determinations of the transcriptional activity under the given experimental conditions. The average coefficient of variation at each hormone concentration was less than 12%. *NR*, no receptor; –, absence of hormone; +, presence of hormone. (Reproduced with permission from Giangrande et al. 1997)

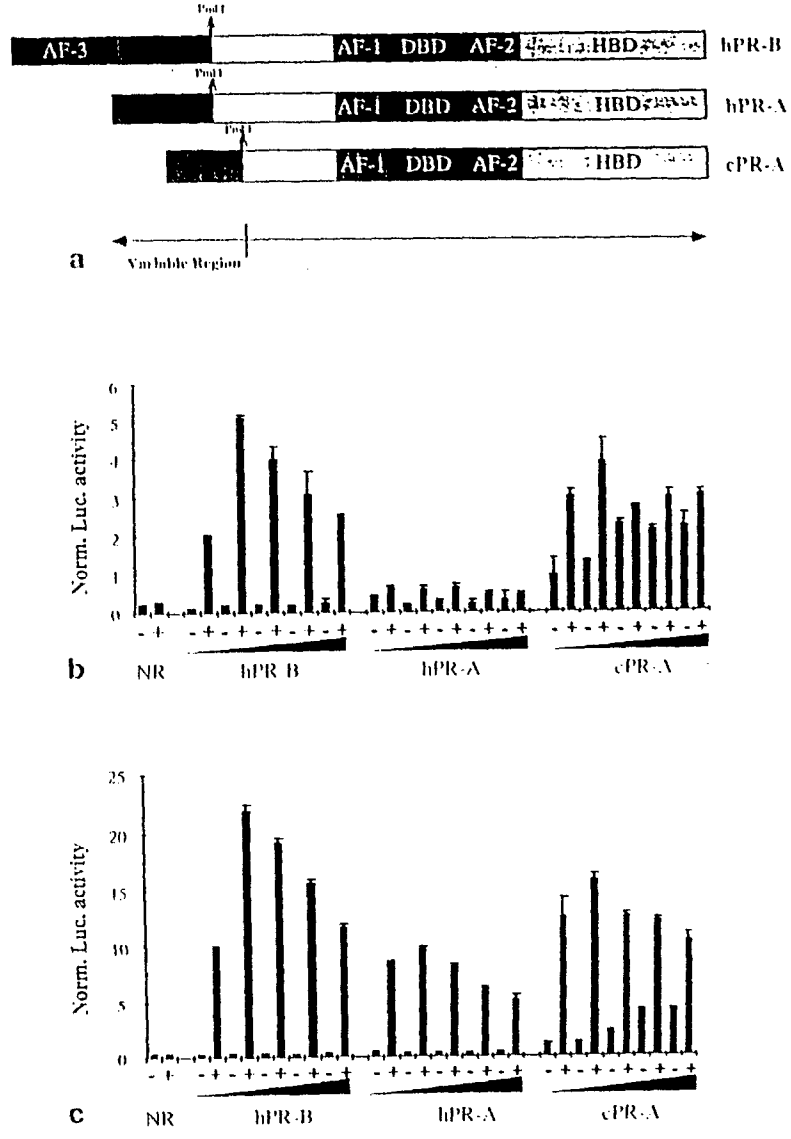


Fig. 1a–c. Legend see p.182

receptors. As expected, these studies indicated that the transcriptional activities of hPR-A and hPR-B were not identical. Surprisingly, however, it was observed that, unlike the case for cPR, hPR-B was an efficient activator of reporter genes containing classical PREs in most contexts examined, whereas hPR-A was transcriptionally inactive (Tung et al. 1993; Vegeto et al. 1993). A representative experiment from these studies is shown in Fig. 1. In this series of experiments the transcriptional activity of hPR-A, hPR-B, and cPR-A were compared in two different cell lines. In HeLa cells, it was observed that hPR-B and cPR-A, but not hPR-A, can function as efficient activators of a progesterone-responsive promoter (Fig. 1b). The result obtained in this cell line is representative of most cells examined. However, in a few cell backgrounds, HepG2 (Fig. 1c) and Ros17.2 (not shown), it was observed that hPR-A can function as an activator. Two important conclusions were drawn from these results: (1) The transcriptional activity of hPR-A and hPR-B were different, and (2) although chickens and humans both express a structurally similar form of PR-A they are not functionally equivalent.

10.4 hPR-A Functions as a Transdominant Repressor of Steroid-Receptor Transcriptional Activity

There are several examples of single genes encoding multiple forms of a single transcription factor (Desombres and Schibler 1991; Foulkes and Sassone-Corsi 1992). One of the most relevant, from the perspective of PR, was the finding that the transcriptional regulator LAP was coexpressed in target cells with a shorter form, LIP. These proteins are produced from a single mRNA as a consequence of alternate initiation of translation. As with hPR-A and hPR-B, it was shown that LIP and LAP were not functionally identical (Desombres and Schibler 1991). Specifically, it was shown that the shorter LIP protein was actually an inhibitor of LAP action and that its expression level in the cell was an important determinant of LAP biology. This observation prompted us to test whether hPR-A could function similarly and regulate hPR-B transcriptional activity. This analysis revealed that, in cells where hPR-A had no inherent transcriptional activity, it actually functioned as a ligand-dependent inhibitor of hPR-B transcriptional activity (Vegeto et al. 1993). This result implied therefore that the primary role of hPR-A was to regulate hPR-B transcriptional activity and that alterations in the relative expression of the two isoforms would be important in determining PR pharmacology.

Although the role of hPR-A as a modulator of hPR-B signaling was predictable based on the established paradigm of LAP and LIP, it was a surprise when it was observed that hPR-A also functioned to regulate the activity of other steroid receptors. This awareness came from a series of control experiments which were designed to test the specificity of hPR-A's inhibitory activity. However, the surprising result of these studies was that co-expression of hPR-A led to the inhibition of the transcriptional activity of the steroid hormone receptors [estrogen receptor (ER); androgen receptor (AR); glucocorticoid receptor (GR); and mineralocorticoid receptor (MR)] but not of other members of the nuclear receptor superfamily (McDonnell and Goldman 1994; McDonnell et al. 1994; Tzukerman et al. 1994; Wen et al. 1994). Of particular importance was the finding that hPR-A, in the presence of agonists, or antagonists, could completely inhibit estradiol-activated ER-mediated transcriptional activity (McDonnell and Goldman 1994). A representative example of this activity is shown in Fig. 2. In cells expressing ER under the control of a constitutive viral promoter, we observed that progesterone alone had no effect on estradiol-activated transcription. However, when hPR-A was co-expressed in the cell, it was observed that ER transcriptional activity was suppressed in a ligand-dependent manner. Interestingly, the inhibitory activity of hPR-A was induced by either agonists or antagonists (Vegeto et al. 1993; McDonnell and Goldman 1994; Wen et al. 1994). The specificity of this response was demonstrated by showing that hPR-B was unable to modulate ER transcriptional activity (McDonnell and Goldman 1994). Although these results were initially puzzling they suggested a mechanism by which the PR and ER signaling pathways could be integrated. Physiologically, progesterone is the natural antagonist of estrogen-mediated processes, an activity which most believe to be related to its ability to down-regulate ER. However, in these studies where ER is expressed from a constitutive viral promoter, we can demonstrate an antiestrogenic activity of PR ligands under conditions where ER expression is not effected. Consequently, these findings indicate that, in addition to receptor regulation, a more complicated molecular mechanism permitting PR-ER crosstalk is

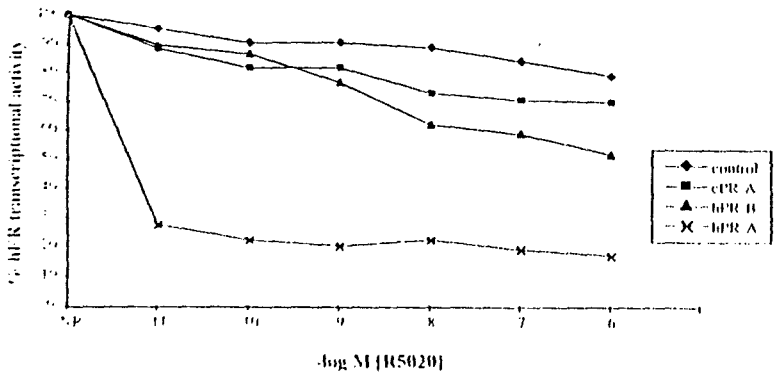
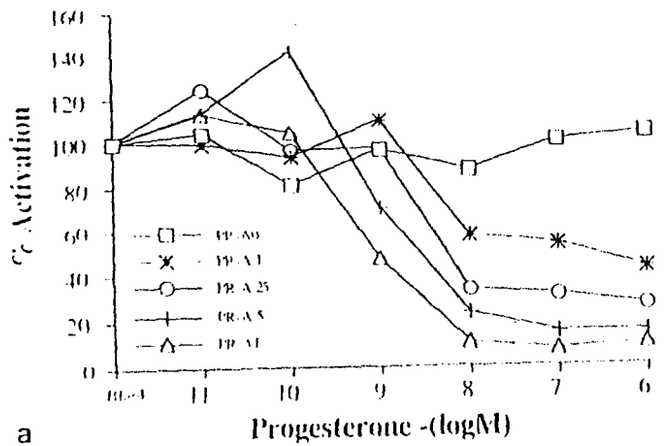


Fig. 2. Transdominant repressor effect of human progesterone receptor A (*hPR-A*), but not human progesterone receptor B (*hPR-B*), or chicken progesterone receptor A (*cPR-A*), on human estrogen receptor (*hER*) transcriptional activity. HeLa cells were transiently transfected with vectors expressing hER alone or in combination with a vector expressing hPR-A (pBK-hPR-A), hPR-B (pBK-hPR-B), or cPR-A (pBK-cPR-A), respectively. The vector pBK-hPR-B was modified by mutating the second in-frame ATG which could potentially yield the A-form of PR. This allows for the expression of hPR-B alone. The transcriptional activity of these constructs was measured following the addition of 10^{-7} M 17β-estradiol alone or in combination with increasing concentrations of R5020 (ranging from 10^{-11} to 10^{-6} M), a progesterone synthetic analogue. In these experiments, ER transcriptional activity was assayed on an ERE2-TATA-LUC reporter. Transfections were normalized for efficiency using an internal β-galactosidase control plasmid (pBK-βgal). The data are presented as percentages of activation, where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the presence of a control vector, pBK-Res-TUP1 (*diamonds*), or in the presence of hPR-A (*X*), hPR-B (*triangles*), or cPR-A (*squares*), respectively, all in the absence of added PR ligands. This value is independently calculated for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation at each hormone concentration was <10%. (Reproduced with permission from Giangrande et al. 1997)

also likely to be operative. This hypothesis, based primarily on the *in vitro* data presented above, was supported by studies in the rat performed by other groups where it was shown that the PR antagonist, RU486, could inhibit estradiol-activated transcription without altering ER expression levels (Kraus and Katzenellenbogen 1993). It appeared from these data, therefore, that the A form of PR was a key modulator of ER pharmacology. Although it was shown that GR, MR, and AR transcriptional activity was also affected by hPR-A expression, the physiological significance of this activity awaits a determination of whether these receptors, and hPR-A, are co-expressed in target cells. Based on our current knowledge of tissue distribution of the steroid hormone receptors, it is likely that the most significant activity of hPR-A will be observed in reproductive tissues where ER- and hPR-B-mediated signaling pathways are both operative.

10.5 The Mechanism of hPR-A-Mediated Inhibition of Steroid Receptor Signaling

The observation that hPR-A inhibited the transcriptional activity of all members of the steroid hormone receptor superfamily suggested that hPR-A was able to inhibit some common pathway or process. Based on the state of the art at the time we considered that "squelching" or titration of a limiting factor required for steroid receptor action was the most likely mechanism. This possibility was addressed in a series of studies which probed the mechanism of hPR-A-mediated inhibition of ER transcriptional activity (Wen et al. 1994). In the absence of expressed hPR-A the transcriptional activity of ER was not affected by the addition of any PR agonists or antagonists (Fig. 3). In contrast, however, the introduction of hPR-A permitted the antiestrogenic activity of these compounds to be manifested. In support of a "squelching" model it was shown that the ability of hPR-A to inhibit hER transcriptional activity was related in a direct manner to its expression level (Fig. 3). However, when we tried to reverse the inhibitory activity of hPR-A by overexpressing hER it was determined that inhibition appeared to depend on the absolute expression level of hPR-A within the cell (Fig. 4). This indicated that squelching, a competitive phenomenon, was not involved, but rather that hPR-A was functioning in an indirect, non-competitive



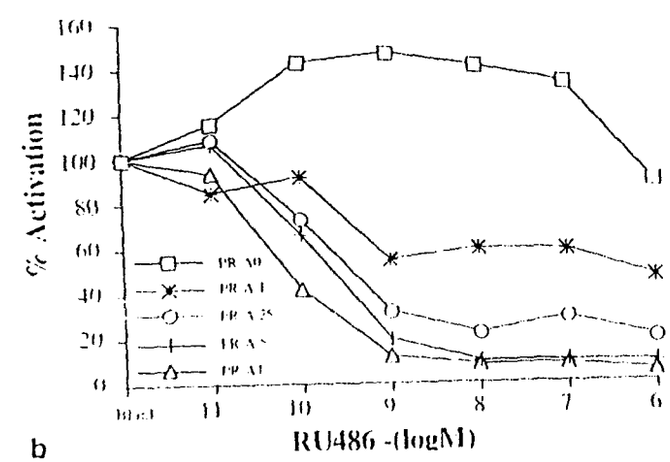
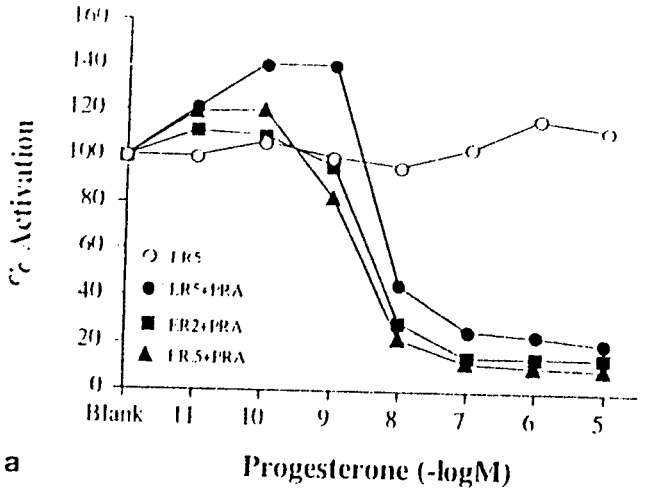


Fig. 3a,b. Legend see. p. 189

manner (Wen et al. 1994). In support of this mechanistic distinction, it has been observed that receptor squelching can be overcome, at least in part, by overexpression of either the steroid receptor co-activator, SRC-1, or the receptor potentiating factor, RPF-1 (Oñate et al. 1995; Imhof and McDonnell 1996). Importantly, however, this manipulation has no effect on the ability of hPR-A to function as a transdominant inhibitor (our unpublished results). We have incorporated data into a working model to explain transdominant inhibitory activity of hPR-A on hER transcriptional activity (Fig. 5). Specifically, we propose that in order for hER to activate transcription it must contact the general transcription apparatus (GTA). In this model, it is implied that this interaction requires the recruitment of at least one, but possibly several, co-activators (X and Y). Data from our studies and those of others suggest that hPR-A can prevent the assembly of a productive ER-GTA complex in either of two ways. One possibility is that the hPR-A dimer binds to the co-activator Y and prevents the ER-co-activator complex from contacting the transcription apparatus (Fig. 5a). Thus, hER and hPR-A manifest their respective activities through different proteins. A second alliterative suggests that both hER and hPR-A can interact with the same co-activator (X) however, their binding sites are not mutually exclusive. The binding of hPR-A in this model would also prevent the association of

◀ **Fig. 3a,b.** Inhibition of human estrogen receptor (hER) transcriptional activity by progesterone receptor (*PR*) ligands is influenced by hPR-A expression level. The effects of increasing cellular concentrations of hPR-A on hER-mediated transcriptional activity were measured in CV-1 cells. An expression vector encoding hER (pRST7hER) (5 μg/ml) was transfected into CV-1 cells alone or in the presence of different concentrations of an hPR-A expression plasmid as indicated. All transfection mixes contained an ERE-TK-LUC reporter (10 μg/ml) (25) and pCH110 (5 μg/ml) as an internal control. The transcriptional activity under these conditions was measured following the addition of 10^{-7} M 17-β-estradiol alone or estradiol in the presence of increasing concentrations of progesterone (a), or RU486 (b), as indicated. Following incubation, cells were harvested and luciferase and β-galactosidase activities were measured. The data are presented as percentages of activation, 100% representing the activity of hER in each condition in the absence of any added PR ligand. Each datum point represents the average of triplicate determinations of the transcriptional activity under a given experimental condition. The average coefficient of variation at each hormone concentration was <15% in this experiment. (Redrawn with permission from Wen et al. 1994)



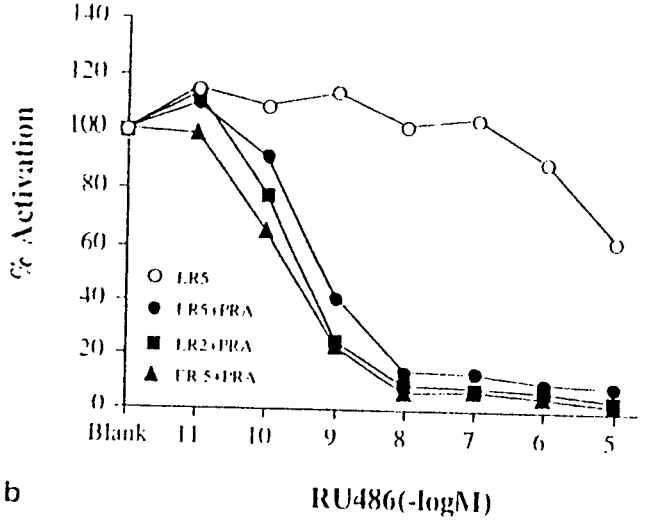


Fig. 4a,b. Legend see p. 191

the ER–co-activator complex with the general transcription apparatus (Fig. 5b). In these proposed pathways repression could result from a simple steric inhibition of transcription, the recruitment by hPR-A of a transcriptional repressor, or a combination of both. The identification of the proteins which specifically interact with hER and hPR in these complexes will be required to resolve this issue.

◀ **Fig. 4a,b.** Inhibition of human estrogen receptor (*hER*) transcriptional activity by subsaturating concentrations of human progesterone receptor A (*hPR-A*) is independent of hER expression level. Monkey kidney CV-1 cells were transiently transfected with increasing concentrations of an hER expression plasmid (as indicated) alone or in the presence of a vector expressing hPR-A. The concentration of hPR-A expression vector (0.5 µg/ml) was shown previously to be submaximal for hPR-A-mediated repression of hER activity. Each transfection condition included a mouse mammary tumor virus-estrogen response element-luciferase (MMTV-ERE-LUC) reporter plasmid (10 µg/ml) and plasmid pCH110 (5 µg/ml) as an internal control. The transcriptional activity in these setups was measured following the addition of 10^{-7} M 17-β-estradiol alone or estradiol in the presence of increasing concentrations of progesterone (**a**), or RU486 (**b**), as indicated. Following incubation, the cells were harvested and luciferase and β-galactosidase activities were measured. The data are presented as percentages of activation; the 100% value represents the activity of hER in each condition in the absence of any added PR ligand. To confirm that the range of ER expression vector chosen allowed an examination of hPR-A activity in the presence of subsaturating and saturating levels of ER, we calculated the fold induction by estradiol in each transfection. The fold inductions in the experiments detailed in (**a**) and (**b**) were as follows: 5 µg of ER expression vector, 31, 26, and 35, respectively; 2 µg of hER expression vector, 28, 24, and 33, respectively; 0.5 µg of hER expression vector, 18, 16, and 24, respectively. Each datum point shown represents the average of triplicate determinations of the transcriptional activity under a given experimental condition. The average coefficient of variation at each hormone concentration was <15% in this experiment. The data presented represent several individual experiments

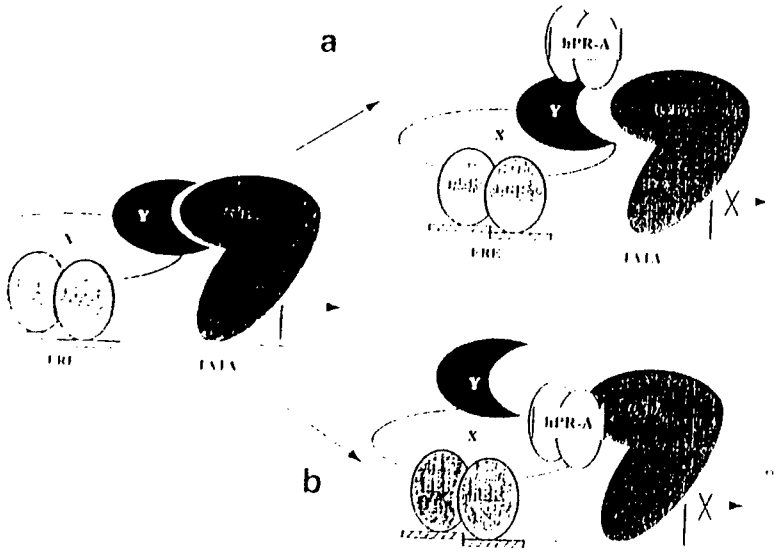


Fig. 5a,b. Human progesterone receptor A (*hPR-A*) functions as a transdominant inhibitor of human estrogen receptor (*hER*) function. Two working models detailing possible mechanisms by which hPR-A can function as a transdominant repressor of hER function are presented. Our data and that of others suggest that ER enhances gene transcription by recruiting one or more transcriptional coactivators to a target gene promoter and permitting the indirect interaction of the receptor with the general transcription apparatus (*GTA*). Two similar, though distinct, mechanisms by which hPR-A can inhibit hER transcriptional activity are proposed: a One possibility is that hPR-A and hER do not interact with the same target proteins. Specifically hER, or other steroid receptors, can interact with their respective coactivator proteins (*X*) but this interaction is not productive because it is blocked hPR-A acting through a distinct target protein (*Y*). b A second plausible model is that hER and hPR-A interact with distinct sites on the same target protein, but that binding of the two proteins is not mutually exclusive. However, the interaction of hPR-A with this target inhibits the positive effect of hER by interrupting the interaction of the receptor-coactivator complex with the transcription apparatus. *ERE*, estrogen response element

10.6 Identification of a Distinct Inhibitory Domain Within hPR-A Required for its Ability to Inhibit Steroid Receptor-Mediated Transcription

It has been assumed that the 164 amino acid hPR-B specific region within PR contains a domain responsible for the functional differences between the two PR isoforms. Indeed, some work has suggested that a distinct activation domain, B upstream sequence (BUS), does in fact exist within this domain (Sartorius et al. 1994). Unfortunately, however, the role of BUS is unclear as it has only been shown to function as an autonomous activator when fused to the PR DNA-binding domain and not other heterologous DNA-binding domains. It has yet to be determined if it will actually function as an activator within the context of the full length PR-B. Another way of explaining the differential activities of hPR-A and B is that both receptors have all the information required to permit the receptors to function as transcriptional activators but that in the context of hPR-A this function is suppressed. This hypothesis implies that sequences which are specific to hPR-B may not necessarily constitute a bona fide activation function, but rather they permit the receptor to overcome the activity of an inhibitory domain within the regions of the receptor common to both isoforms (Kastner et al. 1990). To examine this possibility, we took advantage of the observation that cPR-A, although similar in sequence and structure to hPR-A, functioned as an activator and not an inhibitor of progesterone-responsive genes (Krust et al. 1986; Conneely et al. 1987; Misrahi et al. 1987; Giangrande et al. 1997). Using this information, a series of chimeric proteins in which the least conserved regions of the chicken and the human receptors were produced and analyzed in transfected mammalian cells and examined for their ability to inhibit ER-mediated transcription (Fig. 6). This exercise permitted the mapping of a specific inhibitory domain within hPR-A. Specifically, it was demonstrated that the amino terminal 140 amino acids of hPR-A were necessary for its ability to function as a transcriptional inhibitor. Deletion of this region from hPR-A resulted in a receptor mutant which was functionally indistinguishable from hPR-B (Giangrande et al. 1997). In addition, when the corresponding domains within the human and chicken receptors were exchanged it was determined that the chicken–human receptor chimera could activate transcription, whereas the corollary human–chicken receptor chimera was

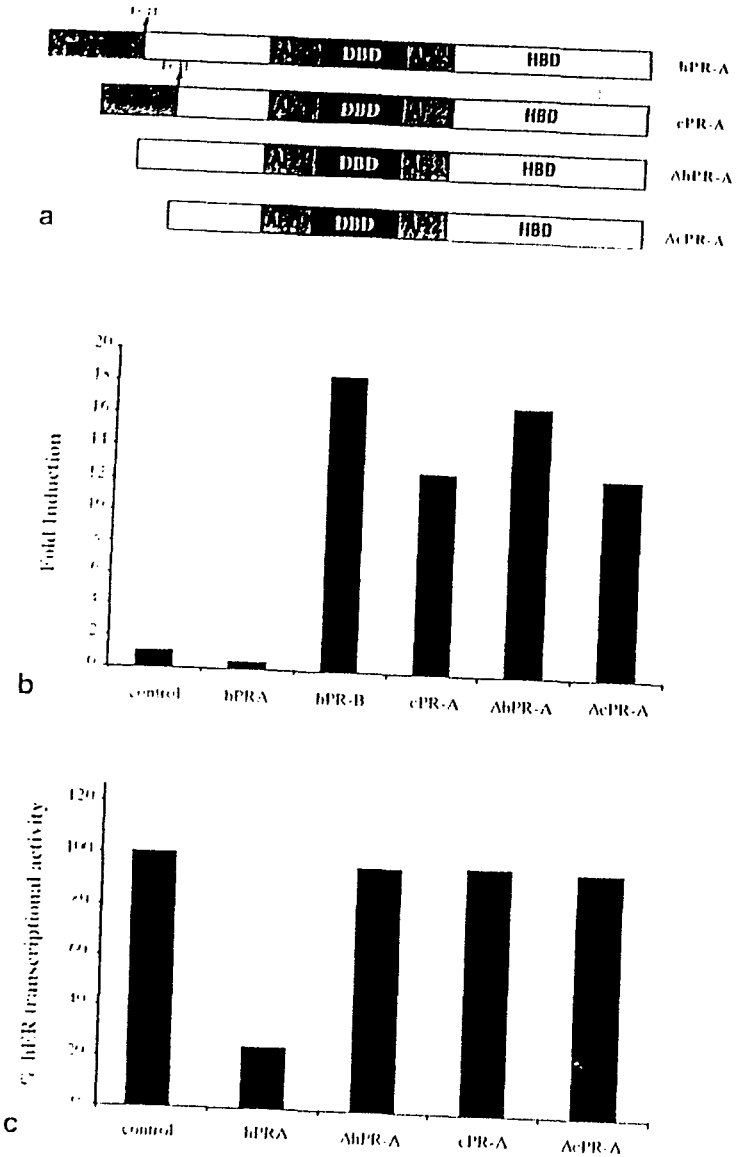


Fig. 6a–c. Legend see p. 195

now a transdominant repressor (Fig. 7). Although these 140 amino acids function as a transferable repression domain they are not able to function as an autonomous repressor when linked to the heterologous GAL4 DNA-binding domain (DBD). Cumulatively, however, these results support the hypothesis that all the sequences required for PR transcriptional activity lie within the region common to hPR-A and hPR-B. However, the inhibitory activity of the amino terminal 140 amino acids of hPR-A masks this activity. We propose, therefore, that the role of the B-specific 164 amino acids is to override the activity of the hPR-A inhibitory domain and permit transcriptional activation.

◀ **Fig. 6a–c.** A human progesterone receptor A (*ΔhPR-A*) is unable to repress human estrogen receptor (*hER*) transcriptional activity. **a** The DNA sequences of hPR-A and chicken PR-A (*cPR-A*) were obtained from GenBank. These represent the full-length sequences of the two receptors. *ΔhPR-A* and *ΔcPR-A*, subcloned into pBK-CMV mammalian expression vector, were generated by deleting the 140 amino acids of hPR-A upstream of the PmlI restriction site and the corresponding 90 amino acids of cPR-A, respectively. *AF-1/AF-2*, activation functions 1 and 2; *DBD*, DNA-binding domain; *HBD*, hormone-binding domain. **b** HeLa cells were transiently transfected with vectors expressing hPR-A, ΔhPR-A, cPR-A, or ΔcPR-A, respectively. The transcriptional activity was measured following the addition of 10^{-7} M R5020. A control vector (pBK-RevTUPT) was used to assess the basal level of transcription of the PRE2-TK-LUC reporter. Transfections were normalized for efficiency using the internal pBK-βgal control plasmid. The data are represented as fold induction, a measure of ligand-induced activity divided by basal (no hormone) activity, for each data point. **c** HeLa cells were transiently transfected with vectors expressing hER alone or in combination with a vector expressing hPR-A, ΔhPR A, cPR-A, or ΔcPR-A, respectively. The transcriptional activity was measured following the addition of 10^{-7} M 17β-estradiol and 10^{-7} M R5020 alone or in combination. In these experiments ER transcriptional activity was assayed on an ERE$_2$-TATA-LUC promoter. Transfections were normalized for efficiency using an internal β-galactosidase control plasmid. The data are presented as percentages of activation, where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the presence of control vector alone or in the presence of hPR-A, ΔhPR-A, cPR-A, and ΔcPR-A, respectively, but in the absence of R5020. This value is independently calculated for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation was <10%. (Reproduced with permission from Giangrande et al. 1997)

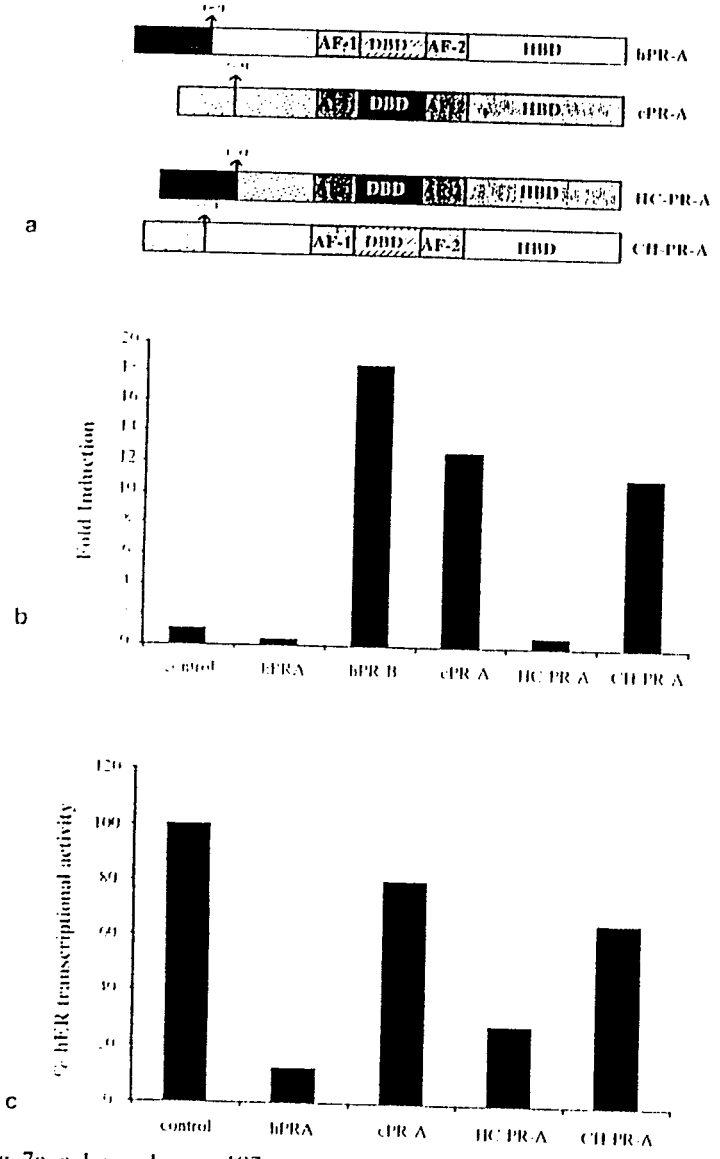


Fig. 7a–c. Legend see p. 197

10.7 Final Comments

The finding that the transcriptional activity of PR-A and PR-B are different and that the influence of PR-A extends to other steroid receptors, begs a redefinition of the term progesterone-responsive genes. Additionally, it suggests that in addition to the expression level of PR-A and PR-B within target cells, alterations in the factors which influence the activities of the two receptor isoforms will have a profound effect on PR pharmacology. These studies have led to the mapping of an inhibitory domain which appears to be responsible for the unique activity of hPR-A. The next step in defining the physiological relevance of this unique activity is to identify the cellular components responsible for distinguishing between hormone-activated hPR-A and hPR-B – an investigative avenue already being carefully explored in several laboratories.

◀ **Fig. 7a–c.** The human chimeric progesterone receptor A construct (*HC-PR-A*) is a potent repressor of human estrogen receptor (*hER*) transcriptional activity in HeLa cells. **a** The human/chicken chimeric constructs (*HC-PR-A* and *CH-PR-A*, respectively) were generated by swapping the N-terminal regions upstream of the unique PmlI restriction site, present in both receptors of the human and chicken receptors. **b** HeLa cells were transiently transfected with vectors expressing human/chicken progesterone receptor A (*hPR-A* and *cPR-A*, respectively) HC-PR-A, and CH-PR-A, respectively. The transcriptional activity of these chimeric constructs was assayed on the PRE_3-TK, progesterone-responsive promoter. The activity was measured after 24-h induction with 10^{-7} M R5020. Fold induction represents the normalized luciferase activity divided by basal (no hormone) activity, for each receptor type after induction with ligand. **c** HeLa cells were transiently transfected with vectors expressing hER alone or in combination with a vector expressing hPR-A, cPR-A, HC-PR-A, and CH-PR-A, respectively. The transcriptional activity was measured following the addition of 10^{-7} M 17β-estradiol and 10^{-7} M R5020 alone or in combination. A control was carried out in the absence of ligands. In these experiments ER transcriptional activity was assayed on an ERE$_2$-TATA-LUC promoter. Transfections were normalized for efficiency using an internal β-galactosidase control plasmid. The data are presented as percentages of activation, where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the presence of hPR-A, cPR-A, HC-PR-A, and CH-PR-A, respectively, but in the absence of R5020. This value is independently calculated for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation was < 11% for both experiments (Reproduced with permission from Giangrande et al. 1997)

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Mapping and Characterization of the Functional Domains Responsible for the Differential Activity of the A and B Isoforms of the Human Progesterone Receptor*

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Paloma H. Giangrande, Giuseppe Pollio, and Donald P. McDonnell‡

From the Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

In humans, the biological response to progesterone is mediated by two distinct forms of the progesterone receptor (human (h) PR-A, 94 kDa and hPR-B, 114 kDa). These two isoforms are transcribed from distinct estrogen-inducible promoters within a single copy PR gene; the only difference between them is that the first 164 amino acids of hPR-B (B-upstream sequence) are absent in hPR-A. In most cell lines such as MCF-7 (human breast cancer cells), CV-1 (monkey kidney fibroblasts), and HeLa (human cervical carcinoma cells), hPR-A functions as a transcriptional repressor, whereas hPR-B functions as a transcriptional activator of progesterone-responsive genes. Interestingly, in these cell contexts, hPR-A also acts as a trans-dominant repressor of the transcriptional activity of other steroid hormone receptors.

In contrast to hPR-A, which functions predominantly as a ligand-dependent transcriptional repressor, we show in this study that the A isoform of the chicken PR (cPR-A) lacks this trans-dominant repressor function and is a transcriptional activator in all contexts examined. By constructing chimeras between the N-terminal domains of the chicken and human PR, we mapped the trans-dominant repressor function of hPR-A to the first 140 amino acids of the protein. Notably, when this 140-amino acid "repressor" domain is placed onto chicken PR-A, the activity of the latter changes from a transcriptional activator to a repressor. Interestingly, however, this "repressor domain" is necessary, but not sufficient, for trans-repression as it is inactive when it is tethered to a heterologous protein. This suggests that the trans-repression function is comprised not only of the repressor domain of hPR-A but also requires the context of the receptor to function. The identification of a discrete inhibitory region within hPR-A which is transferable to another receptor implies that this region interacts with a set of transcription factors or adaptors that are distinct from those recognized by hPR-B, the identification of which will be required to define the mechanism by which hPR-A modulates steroid hormone receptor transcriptional activity. Thus, although chickens and humans both produce two very similar forms of the progesterone receptor, it is clear from these studies that the mechanism of action of progesterone in these two systems is quite different.

The progesterone receptor (PR)[1] belongs to the superfamily of intracellular receptors that mediate the nuclear effects of steroid hormones, thyroid hormone, and the non-nutritional vitamins A and D (1). The mechanism of action of PR is similar to that of the other steroid receptors. In the absence of ligand the receptor is transcriptionally inactive and remains sequestered in a large complex of heat shock proteins (HSPs) as follows: HSP-90, HSP-70, and P59 (2–4). Upon ligand binding, the receptor undergoes a distinct change in conformation (5) that results in the dissociation of a monomeric receptor from the heat shock complex (5, 6). Liganded receptors then spontaneously dimerize and bind to DNA via specific progesterone response elements (PREs) located within the regulatory regions of target genes (7). The binding of either an agonist, or of most antagonists, converts the receptor into a DNA binding competent form (8). However, only agonist-bound PR receptors are capable of enhancing transcriptional activation when bound to PREs.

The progesterone receptor structure is similar to that of other steroid receptors in that it contains a highly conserved DNA-binding domain (DBD), a hormone-binding domain (HBD) (conserved among the related steroid receptors such as PR and glucocorticoid receptor (GR)), and an N-terminal domain which is the most variable region among the family members (9). The regions responsible for receptor dimerization and interaction with heat shock proteins are also located at the C terminus within the HBD of PR (10). More importantly, the HBD also contains one of the transcriptional activation domains AF-2 (11). The other transcriptional activation domain, AF-1, is located in the N terminus upstream of the DBD (9, 12).

The human PR is unique in that it exists as two isoforms hPR-B (114 kDa) and hPR-A (94 kDa) (13). The human PR-A is a truncated form of hPR-B lacking the first 164 N-terminal amino acids. These two isoforms are transcribed from distinct estrogen-inducible promoters within a single copy PR gene (14). Both isoforms have been identified in most species, with the exception of the rabbit where PR exists only as the B isoform (15). The biochemical properties of the two PR isoforms have been analyzed extensively *in vitro*. Both forms have similar DNA and ligand binding affinities (16). However, work done using reconstituted progesterone-responsive transcription sys-

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‡ To whom correspondence should be addressed: Dept. of Pharmacology and Cancer Biology, Box 3813 Duke University Medical Center, Durham, NC 27710. Tel.: 919-684-6035; Fax: 919-681-7139; E-mail: mcdon016@acpub.duke.edu.

[1] The abbreviations used are: PR, progesterone receptor; c, chicken; h, human; HSP, heat shock protein; PRE, progesterone response elements; DBD, DNA-binding domain; HBD, hormone-binding domain; GR, glucocorticoid receptor; ER, estrogen receptor; ERE, ER element; PCR, polymerase chain reaction; CMV, cytomegalovirus; BUS, B-upstream segment; 17β-E_2, 17β-estradiol.

tems in various mammalian cells revealed that hPR-A and hPR-B are not functionally identical (9, 14, 17). Specifically, hPR-B functions as a transcriptional activator in most contexts, whereas in most cells hPR-A does not activate transcription but functions as a strong trans-dominant repressor of hPR-B, glucocorticoid receptor (hGR), androgen receptor, mineralocorticoid receptor, and estrogen receptor (hER) transcriptional activity (17–19).

Unlike the human receptor, both the A and B isoforms of the chicken PR act as potent activators of progesterone-responsive genes in transfected mammalian cells (20–22). Thus, although the primary sequences of the cPR-A and hPR-A are quite similar, they are functionally quite different. A comparison of the amino acid sequence revealed that cPR-A and hPR-A are very homologous over most of the length of the protein; however, they are divergent in their N termini. Consequently, we hypothesized that the key sequences responsible for the different activities of cPR-A and hPR-A lie within the N terminus. We anticipated that by creating and analyzing chimeras between the two proteins that the sequences within hPR-A required for trans-repression could be defined.

In this study, we show that the N-terminal 140-amino acid region of hPR-A is necessary, but not sufficient, for trans-repression of ER transcriptional activity. In addition, our findings indicate that this repressor region of hPR-A requires other domains within the receptor to form the structures necessary for trans-repression. It is possible that this structure is required to sequester a co-factor required for proper hER transcriptional activity.

EXPERIMENTAL PROCEDURES

Materials—DNA restriction and modification enzymes were obtained from Promega (Madison, WI), Boehringer Mannheim, or New England Biolabs (Beverly, MA). PCR reagents were obtained from Perkin-Elmer or Promega Corp. (Madison, WI). Progesterone and 17β-estradiol were purchased from Sigma. R5020 (promegestone) was purchased from NEN Life Science Products. Secondary antibodies, Hybond-C Extra (nitrocellulose) transfer membrane, and developing film were obtained from Amersham Corp. PR22 primary monoclonal antibody was a gift from David Toft (Mayo Clinic, Rochester, MN). Polyclonal antibody raised against hPR-A was a gift from Nancy Weigel (Baylor College of Medicine, Houston).

Plasmids—The expression plasmid CMV-hPR-B was constructed as follows: YEphPR-B (23) was digested with *Xho*I and *Kpn*I, and the fragment containing the coding sequence for hPR-B was ligated into pBK-CMV mammalian expression vector, previously digested with *Xho*I and *Kpn*I (Stratagene, La Jolla, CA). pBKC-hPR-A was constructed as follows: YEphPR-A was digested with *Xho*I and *Kpn*I, and the fragment containing the coding sequence for hPR-A only was ligated into pBK-CMV as described above. YEphPR-A was constructed as follows: YEpE2 (24) vector was digested with *Nco*I and *Kpn*I and subsequent cloning of a PCR-generated fragment from YEphPR-B. The sequences of the oligonucleotides for PCR were 5′-CCGCCCATGGGCCGGTCCGGGTGCAAGG (forward) and 5′-GCCATCTTGGTACCCCG (reverse). pBKC-cPR-A was constructed as follows: an *Eco*RI fragment containing the coding sequence for cPR-A was digested from pADA (25) and ligated into pBSII-KS (Stratagene), shuttle vector. The ligated product was then digested with *Xho*I and *Xba*I, and the fragment containing the cPR-A cDNA sequence was ligated into pBK-CMV, previously digested with *Xho*I and *Xba*I.

The deletion mutant pBKC-ΔhPR-A was constructed as follows: pBKC-hPR-A was digested with *Pst*I and PmLI to delete a 420-base pair fragment. The complementary oligonucleotides, 5′-GGGGCGCGAATTCTCACGGATGCAC (forward) and 5′-GTGCATCCGTGAGAATTCGCGCCCCTGCA (reverse), were annealed and ligated into the vector to create a new translation start site. A unique *Eco*RI site was included in the oligonucleotide sequence to facilitate detection of incorporated oligonucleotides. The deletion mutant pBKC-ΔcPR-A was constructed as follows: pBKC-cPR-A was digested with *Xho*I and PmLI to delete a 270-base pair fragment. The complementary oligonucleotides, 5′-TCGAGCGACGCGTGATACGGATGCAC (forward) and 5′-GTGCATCCGTATCACGCGTCGC (reverse), were annealed and ligated into the vector to create a new translation start site. All deletion mutants were verified by sequencing to ensure the fidelity of the resulting constructs.

The GAL4-DBD fusion constructs were cloned into pBK-CMV mammalian expression vector. The pBKC-DBD plasmid was constructed as follows: a *Bgl*II/*Eco*RI fragment from pSG424 (Stratagene), containing GAL4-DBD, was subcloned into pBK-CMV expression plasmid. pBKC-DBD-HBD was made by subcloning the HBD of PR (obtained from pOPRSVI-PR-A, a pOPRSVI base plasmid containing pBSII-KS MCS with *Bam*HI fragment from YEphPR-B encoding the A-form of hPR a gift from Markus O. Imhof, Swiss Federal Institute of Technology-Lausanne, Lausanne, Switzerland) into *Eco*RI/*Not*I site of pBKC-DBD. The pBKC-DBD-AF1 plasmid was constructed by digesting pBKC-DBD with *Eco*RI and *Xba*I and subsequent cloning of a PCR-generated fragment from pOPRSVI-PR-A. The sequences of the oligonucleotides for PCR were 5′-CCGGAATTCATGTCGACCCTGGAGTGCATCCTG (forward) and 5′-CCCTCTAGATTACCTCAGGTAGTTGAGATAGGGCGG (reverse).

The plasmid pBKC-DBD-NhPR-B was created by digesting pBKC-DBD with *Eco*RI and *Xba*I and subsequent cloning of a PCR-generated fragment from pOPRSVI-hPR-A. The sequences of the oligonucleotides for PCR were 5′-CCGGAATTCGTCATGACTGAGCTGAAGGCAAAGGG (forward) and 5′-CCCTCTAGATTACCTCAGGTAGTTGAGATAGGGCGG (reverse).

The plasmid pBKC-DBD-NhPR-A was created as described above. The sequences of oligonucleotides for PCR were 5′-CCGGAATTCCGGATGAGCCGGTCCGGG (forward) and 5′-CCCTCTAGATTACACGTGGATGAAATC (reverse).

The plasmid pBKC-DBD-140NhPR-A was created as outlined above. The sequences of oligonucleotides for PCR were 5′- CCGGAATTCCGGATGAGCCGGTCCGGG (forward) and 5′-CCCTCTAGATTAGCTGGCTTCTGAATCCGG (reverse).

Site-directed Mutagenesis—The expression vector pBKC-hPR-B which expresses hPR-B only was constructed as follows: the template for the mutagenesis was CMV-hPR-B. The point mutations were created using PCR-based oligonucleotide-directed mutagenesis, according to manufacturers guidelines (Stratagene). This was done by replacing the second ATG, which gives rise to hPR-A transcript, with GCG, thus creating a unique *Nru*I site. This restriction site was used to facilitate the detection of the incorporated mutation. The sequences of the oligonucleotides for PCR were 5′-TGTTGTCCCCGCTCGCGAGCCGGTCCGGGTGCAAG (forward) and 5′-CTTGCACCCGGACCGGCTCGCGAGCGGGGACAACA (reverse). All PCR-based cloning was verified by sequencing to ensure the fidelity of the resulting constructs.

Cell Culture and Transient Transfection Assays—HeLa and HepG2 cells were maintained in modified Eagle's medium (Life Technologies, Inc.) plus 10% fetal calf serum (Life Technologies, Inc.). Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h prior to transfection. DNA was introduced into the cells using Lipofectin (Life Technologies, Inc.). Briefly, triplicate transfections were performed using 3 μg of total DNA. For standard transfections 50 ng of pBKC-βgal (normalization vector) (26), 1500 ng of reporter (either PRE_3-TK-LUC, ERE_3-TATA-LUC, or $GAL4_5$-TATA-LUC), 500 ng of pRST7-ER (27) or control vector pBSII-KS (Stratagene), and variable amounts (corrected for molarity) of receptor (either hPR-A, hPR-B, cPR-A, deletion mutants, or GAL4 fusions). A control pBCK-CMV-based plasmid (pBKC-Rev-TUP1) (a gift from Ben Lieberman, University of Colorado Health Center, Denver, CO) was used to adjust for the total amount of CMV. The reporter ERE_3-TATA-LUC, contains three copies of vitellogenin ERE. The reporter PRE_3-TK-LUC contains three copies of a consensus PRE. The reporter $GAL4_5$-TATA-LUC (generous gift from Dr. Xiao-Fan Wang, Duke University Medical School) contains five palindromic 17-base pair GAL4-recognition sites cloned into pGL2-TATA-Inr (Stratagene). Incubation of the cells with Lipofectin proceeded for 3 h, at which time media were removed and then induced with appropriate hormone diluted in phenol red-free media containing 10% charcoal-stripped fetal calf serum (Hyclone Inc., Logan, UT). Incubation with hormone continued for 24 h, after which cells were lysed and assayed for luciferase and β-galactosidase activity as described previously (28).

RESULTS

Differential Transcriptional Activities of hPR-B, hPR-A, and cPR-A—To compare the transcriptional activities of hPR-B, hPR-A, and cPR-A, we used the expression vectors pBKC-hPR-B, pBKC-hPR-A, and pBKC-cPR-A which specifically encode either hPR-B, hPR-A, or cPR-A. The expression constructs were transiently transfected into HeLa (human cervical carci-

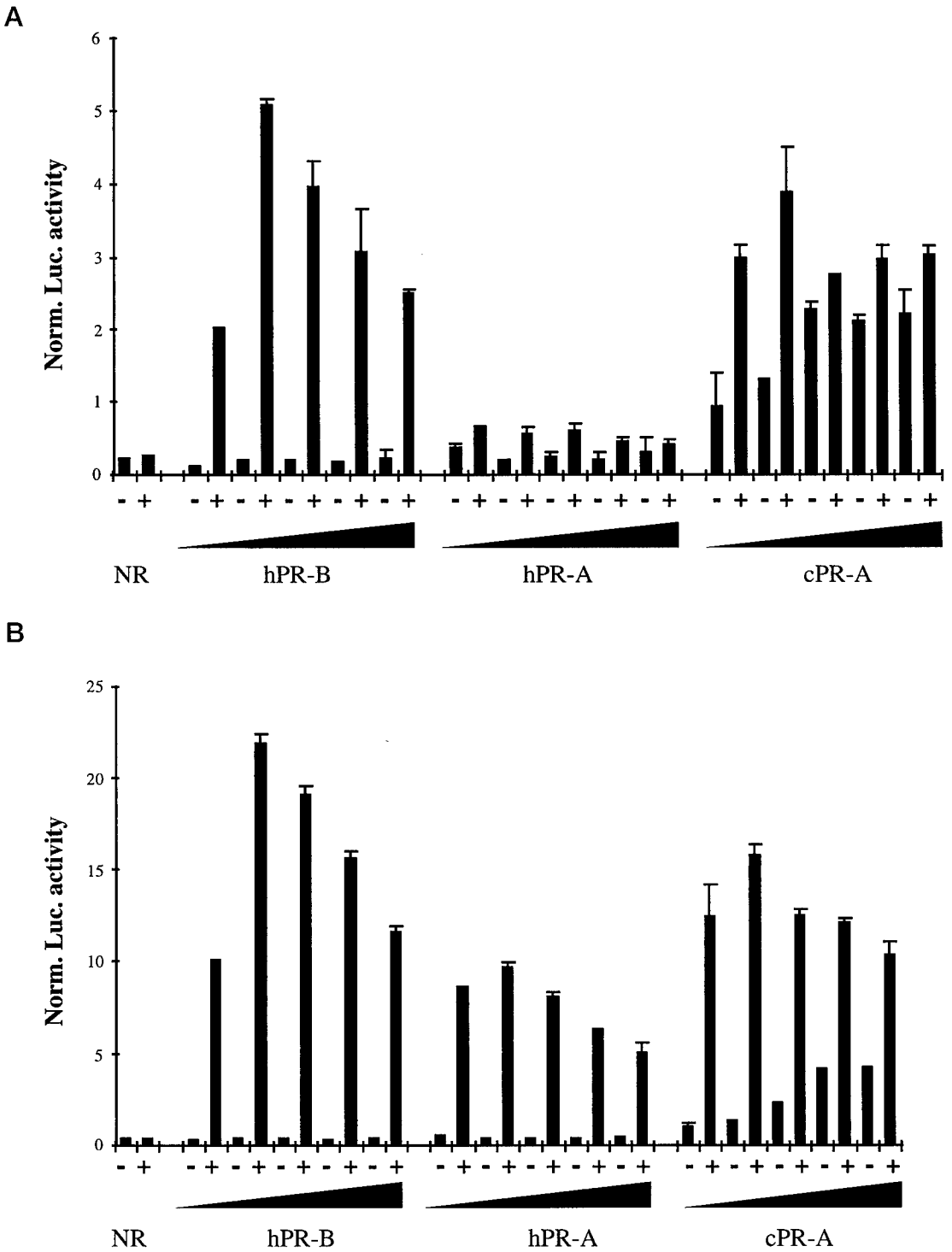

FIG. 1. **Differential transcriptional activities of hPR-B, hPR-A, and cPR-A.** HeLa cells (*A*) and HepG2 cells (*B*) were transiently transfected with increasing concentrations of vectors expressing hPR-B, hPR-A, or cPR-A ranging from 1 to 5 ×, where X represents the respective concentration for each receptor corrected for molarity (where $X = 0.131$ μg for hPR-B, 0.12 μg for hPR-A, and 0.115 μg for cPR-A). The transcriptional activity was measured 24 h after the addition of 10^{-7} M R5020. In these experiments progesterone receptor transcriptional activity was assayed on a PRE_3-TK-LUC promoter (1.5 μg). Transfections were normalized for efficiency using 0.05 μg of an internal β-galactosidase, control plasmid (pBKC-βgal). Luciferase activity (*Luc. activity*) was normalized to β-galactosidase activity. The total concentration of CMV promoter was kept constant throughout the experiment by including the appropriate amount of a CMV-based control plasmid (pBK-Rev-TUP1). The total amount of DNA per triplicate was 3.0 μg. Each data point represents the average of triplicate determinations of the transcriptional activity under the given experimental conditions. The average coefficient of variation at each hormone concentration was less than 12%. *NR*, no receptor; −, absence of hormone; +, presence of hormone.

noma) cells (Fig. 1*A*) or HepG2 (human hepatoma) cells (Fig. 1*B*) together with a progesterone-responsive luciferase reporter (PRE_3-TK-LUC). Western immunoblot analysis using a human PR-specific polyclonal antibody (B13-TK) and a chicken PR-specific monoclonal antibody (PR22) confirmed that the receptors expressed in an intact form at approximately the same level (data not shown).

HeLa cells and HepG2 cells contain no endogenous PRs. As a result, there was no significant hormone-dependent activation of the PRE_3-TK promoter in the absence of transfected receptor

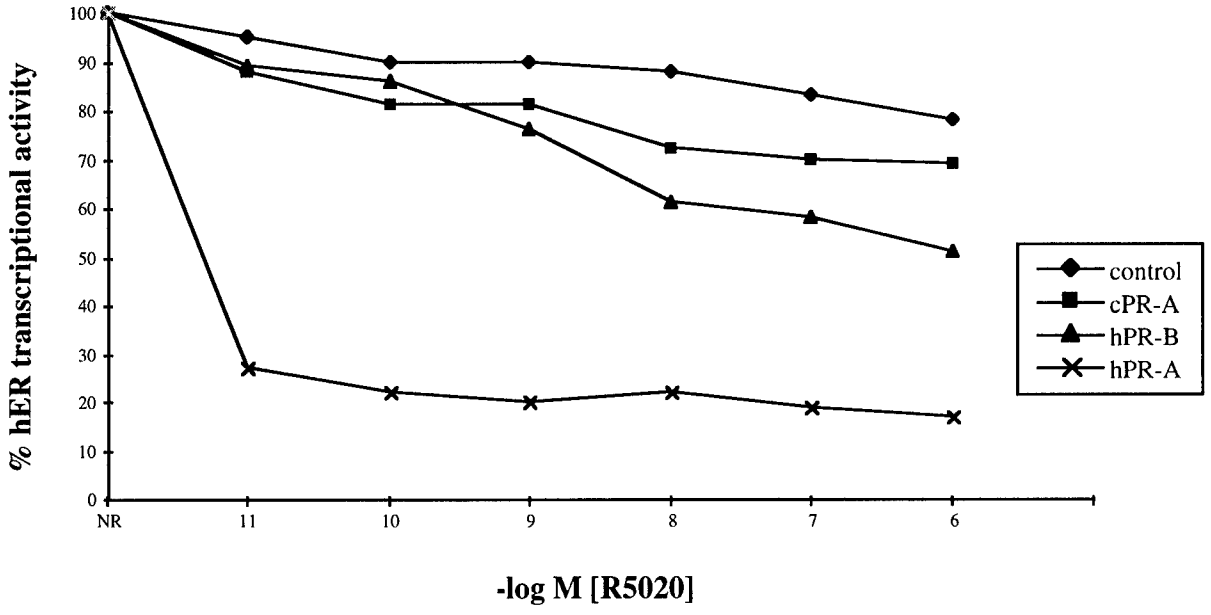

FIG. 2. **Trans-dominant repressor effect of hPR-A, but not hPR-B or cPR-A, on hER transcriptional activity.** HeLa cells were transiently transfected with vectors expressing the human estrogen receptor alone or in combination with a vector expressing hPR-A (pBK-hPR-A), hPR-B (pBK-hPR-B), or cPR-A (pBK-cPR-A), respectively. The vector pBK-hPR-B was modified by mutating the second in-frame ATG which potentially could yield the A-form of PR. This allows the expression of hPR-B alone. The transcriptional activity of these constructs was measured following the addition of 10^{-7} M 17β-estradiol alone or in combination with increasing concentrations of R5020 (ranging from 10^{-11} to 10^{-6} M), a progesterone synthetic analog. In these experiments estrogen receptor transcriptional activity was assayed on a ERE_3-TATA-LUC reporter. Transfections were normalized for efficiency using an internal β-galactosidase control plasmid (pBK-βgal). The data are presented as % activation, where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the presence of a control vector, pBK-Rev-TUP1 (*diamonds*), or in the presence of hPR-A (×), hPR-B (*triangles*), or cPR-A (*squares*), respectively, all in the absence of added PR ligands. This value is independently calculated for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation at each hormone concentration was <10%.

(NR) even upon the addition of ligand (Fig. 1, *A* and *B*). As expected, transfection of increasing amounts of pBKC-hPR-B expression vector in either cell context permitted progesterone-mediated activation of the PRE_3-TK promoter, the degree of which was proportional to the amount of input plasmid. At higher plasmid concentrations, however, we observed a decrease in the transcriptional activity of hPR-B. This is likely due to self-squelching where overexpression of the receptor titrates out a limiting factor (29). In contrast, in HeLa cells but not HepG2 cells no significant progesterone-induced activation of PRE_3-TK promoter by hPR-A was observed. The influence of cell type on the human PR subtype-specific activation of progesterone-responsive promoters has been documented previously (17).

The most striking result, however, was observed when we compared the transcriptional activities of hPR-A and cPR-A. In HeLa cells, as we had observed in the past, hPR-A was only marginally active as a transcriptional activator, at any expression level tested. However, cPR-A demonstrated an activity that was equivalent to hPR-B. Furthermore, at higher receptor concentrations, cPR-A displayed increased ligand-independent activity which was not observed with either isoform of human PR (Fig. 1*A*). This clearly demonstrates a functional difference between the A-form of PR from the two species. Interestingly, this difference was not manifested in HepG2, cells where all three receptor isoforms tested were transcriptionally active. These results confirmed and expanded our previous studies showing that hPR-A and hPR-B were functionally distinct. However, more importantly they indicated that the PR-A isoforms from chicken and human are not functionally equivalent as activators of transcription.

Trans-dominant Repressor Effect of hPR-A but Not hPR-B or cPR-A on hER Transcriptional Activity—Previously, it has been shown that hPR-A but not hPR-B is capable of trans-dominant repression of steroid receptor activity in contexts where it has no independent positive transcriptional activity (17, 19, 30). To determine whether cPR-A is also capable of trans-dominant repression of heterologous steroid receptor action, we transiently transfected into HeLa cells the constructs expressing either hPR-B, hPR-A, or cPR-A together with an estrogen-responsive luciferase reporter (ERE_3-TATA-LUC) and an expression vector for hER (pRST7-ER) (Fig. 2). The experiments were performed using concentrations of hPR-B, hPR-A, and cPR-A which gave the maximal ligand-dependent transcriptional activation (Fig. 1*A*). Estradiol-dependent activation of the ERE_3-TATA promoter in HeLa cells expressing hER together with control plasmid was not affected by co-addition of R5020 at any concentration (ranging from 10^{-11} to 10^{-6} M). However, HeLa cells cotransfected with hPR-A inhibited hER-mediated transcriptional activity by 78% at 10^{-11} M R5020 which increased to >80% with increasing concentrations of R5020 (Fig. 2). In contrast, HeLa cells cotransfected with hPR-B or cPR-A showed little or no trans-dominant repression of hER transcriptional activity. At higher concentrations of R5020 (10^{-6} M) hER activity could be repressed by 45% in the presence of hPR-B but not cPR-A. Importantly, in these experiments cPR-A has no effect on ER transcriptional activity. These data suggest a selective role for hPR-A, but not hPR-B, or cPR-A in the negative regulation of steroid receptor transcriptional activity.

The observation that the structurally related cPR-A and hPR-A proteins have completely different functions suggests to us that by constructing receptor chimeras we would be able to define the regions within hPR-A responsible for trans-dominant repression.

Structural Differences between hPR-B, hPR-A, and cPR-

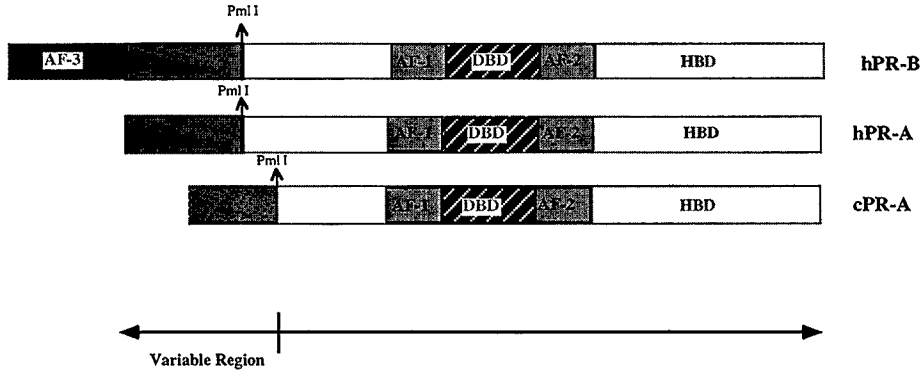

FIG. 3. **Sequence similarities among PR isoforms.** The DNA sequences of the human and the chicken isoforms of the progesterone receptor were obtained from GenBank. Regions of amino acid similarities between hPR-A and cPR-A were determined using the DNA Strider and LALNVIEW programs. The regions of least homology are located upstream of the unique PmLI restriction site present in both receptors (55% similarity, 30% identity). Regions of high homology are found downstream of the PmL1 restriction site (90% similarity, >72% identity). The amino acid sequence of the B isoform of the human progesterone receptor is also detailed above. *hPR-B,* human progesterone receptor-B; *hPR-A,* human progesterone receptor-A; *cPR-A,* chicken progesterone receptor-A; *HBD,* hormone binding domain; *AF-2,* activation function-2; *DBD,* DNA-binding domain; *AF-1,* activation function-1; *AF-3,* activation function-3.

A—It has been postulated that the 164-amino acid B-upstream segment (BUS), unique to hPR-B, is in part responsible for the functional differences between the two isoforms of human PR (29). However, this cannot be the complete answer as our data, and those of others (21, 31), show that unlike its human counterpart the cPR-A is a strong activator of progesterone-responsive promoters, yet it lacks the BUS activating function present in hPR-B. These observations imply that it is some other regulatory element present in hPR-A that is responsible for the differential activities observed with the two isoforms of human PR. We compared the primary structures of hPR-A and cPR-A (Fig. 3) to identify sequences present in hPR-A that may be responsible for its unique inhibitory action. From this comparison we concluded that the most extensive differences in the primary structures of the chicken and human PR-As are found in the N-terminal domains, upstream of a unique PmL1 restriction site present in both receptors. These N-terminal domains of the receptors share only 55% similarity and 30% identity compared with 90% similarity and >72% identity shared between the C-terminal regions of the receptors. Based on this observation we hypothesized that the unique trans-dominant activities of hPR-A were determined by the extreme N-terminal 140 amino acids.

The N-terminal 140-Amino Acid Region of hPR-A Is Necessary for Trans-repression of Heterologous Steroid Receptor Transcriptional Activity—Previous work has mapped a major activation function within hPR to a 90-amino acid region (AF-1), contained within both hPR-A and hPR-B. These studies revealed also that another region wholly contained within the BUS region of hPR-B was required for maximal AF-1 activity (29). This suggested to us that a major role of BUS was to overcome a repressive activity of the N-terminal of hPR-A on AF-1. To address this hypothesis we created a series of deletion mutants that lacked the first 140-amino acids in the human (ΔhPR-A) and the corresponding 90-amino acids in the chicken (ΔcPR-A) upstream of the unique PmL1 site (Fig. 4*A*). We observed that unlike full-length hPR-A, ΔhPR-A acquired the ability to activate progesterone-responsive promoters (Fig. 4*B*). Western immunoblots confirmed equal expression of hPR-A and ΔhPR-A (data not shown). On the contrary, deletion of the first 90 amino acids from cPR-A did not affect its ability to activate progesterone-responsive promoters (Fig. 4*B*). Overall, these results suggest that the inability of hPR-A to function as a transcriptional activator is not due to a loss of an activation sequence in BUS but due to the active inhibitory actions of the N-terminal 140 amino acids of hPR-A.

If the inability of hPR-A to activate transcription is due solely to the inhibitory activity of N-terminal 140 amino acids, then we predicted that hPR-A mutants lacking this activity would be unable to act as trans-dominant repressors. Therefore, to determine the ability of the deletion mutants to trans-repress heterologous steroid receptor activity, we tested the ability of the individual mutants to repress hER transcriptional activity. Vectors expressing hER and either ΔhPR-A or ΔcPR-A, respectively, were cotransfected into HeLa cells (Fig. 4*C*) together with the ERE_3-TATA-LUC reporter. hER transcriptional activity in the presence of 10^{-7} M 17β-E_2 alone or in the presence of 10^{-7} M 17β-E_2 and 10^{-7} M R5020 in combination was measured after 24 h. As shown in Fig. 4*C* wild type hPR-A repressed 17β-E_2-dependent transcription by hER by 80%, whereas ΔhPR-A was unable to repress hER activity under the same conditions. Both cPR-A and the 90-amino acid truncated form of this receptor, ΔcPR-A, displayed no trans-repressive effect on hER transcriptional activity. From these data we concluded that the N-terminal 140 amino acids of hPR-A contain a specific "inhibitory domain" and that this is necessary for trans-dominant repression of hER. Similarly, we also showed that the ability of hPR-A to repress the transcriptional activity of either the human glucocorticoid receptor (hGR) or that of hPR-B required the 140-amino acid hPR-A inhibitory domain (data not shown). We conclude, therefore, that hPR-A-mediated repression of steroid receptor transcriptional activity occurs through a similar mechanism.

To characterize further hPR-A's inhibitory domains and to see whether it was transferable, we swapped the 140 amino acids of hPR-A with the 90 amino acids from cPR-A, to create the chimeras HC-PR-A and CH-PR-A, respectively (Fig. 5*A*). Both chimeric receptors were subcloned into pBK-CMV mammalian expression vectors and were shown by Western immunoblot to be expressed at the same level as their wild type counterparts (data not shown). As expected, when testing the ability of these chimeric fusions to activate progesterone-responsive promoters (PRE_3-TK-LUC), we noticed that like wild type hPR-A, HC-PR-A had no positive transcriptional activity on this promoter, whereas wild type cPR-A was capable of 12-fold activation under these conditions (Fig. 5*B*). These results strongly suggested that the repressor effect observed with hPR-A is transferred along with the N-terminal 140 amino acids of the receptor since deletion of the 90 amino acids of cPR-A has no effect on the ability to transactivate. The chi-

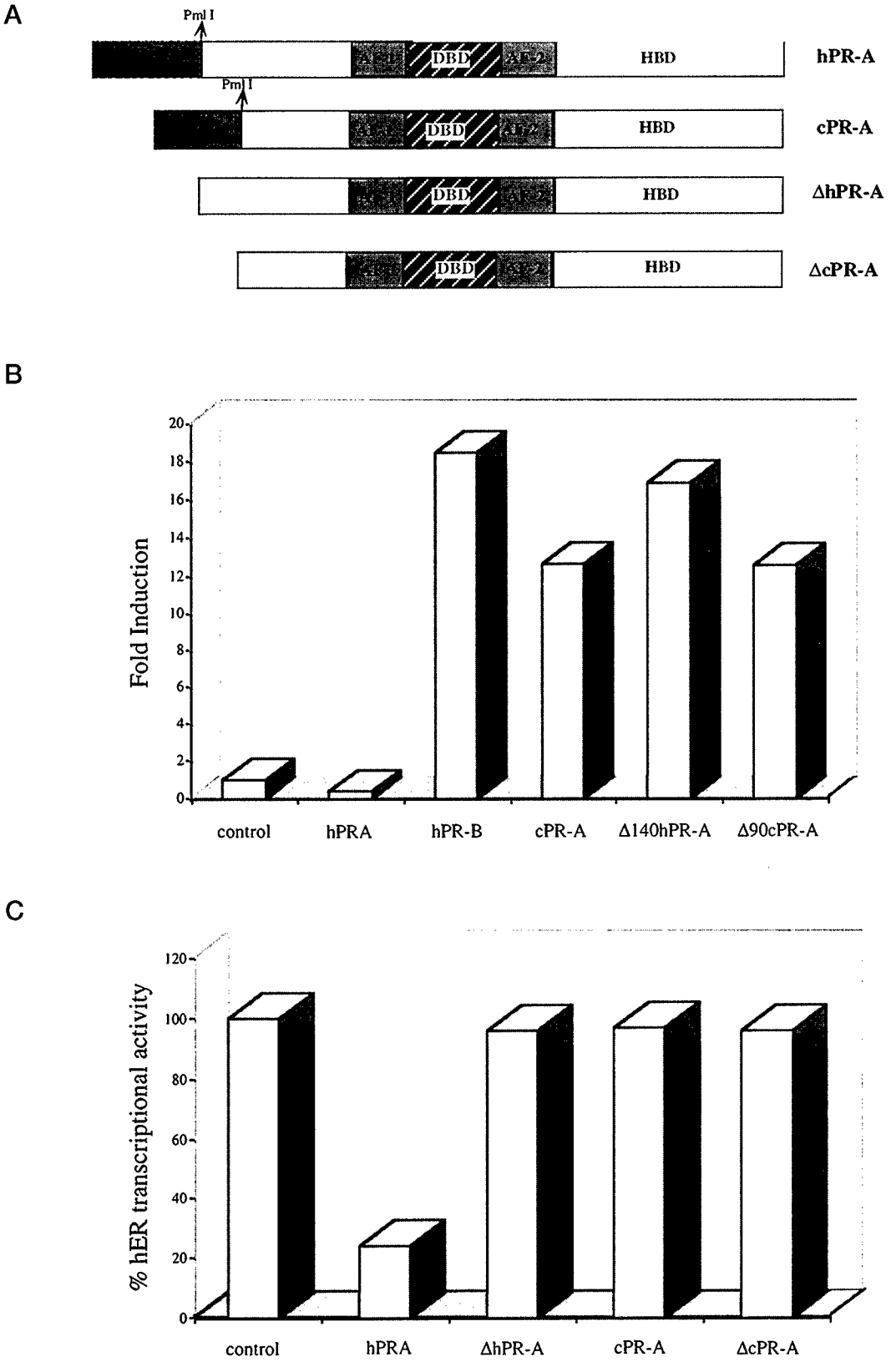

FIG. 4. **ΔhPR-A is unable to repress hER transcriptional activity.** *A*, the DNA sequences of hPR-A and cPR-A were obtained from GenBank. These represent the full-length sequences of the two receptors. ΔhPR-A and ΔcPR-A, subcloned into pBK-CMV mammalian expression vector, were generated by deleting the 140 amino acids of hPR-A upstream of the PmL1 restriction site and the corresponding 90 amino acids of cPR-A, respectively. *B*, HeLa cells were transiently transfected with vectors expressing hPR-A, ΔhPR-A, cPR-A, or ΔcPR-A, respectively. The transcriptional activity was measured following the addition of 10^{-7} M R5020. A control vector (pBK-RevTUP1) was used to assess the basal level of transcription of the PRE_3-TK-LUC reporter. Transfections were normalized for efficiency using the internal pBK-βgal control plasmid. The data are represented as *Fold Induction*, a measure of ligand induced activity divided by basal (no hormone) activity, for each data point. *C*, HeLa cells were transiently transfected with vectors expressing the human estrogen receptor, *hER*, alone or in combination with a vector expressing hPR-A, ΔhPR-A, cPR-A, or ΔcPR-A, respectively. The transcriptional activity was measured following the addition of 10^{-7} M 17β-estradiol and 10^{-7} M R5020 alone or in combination. In these experiments estrogen receptor transcriptional activity was assayed on an ERE_3-TATA-LUC promoter.

meric fusion CH-PR-A was as active as wild type cPR-A, and both were similarly active to wild type hPR-B (Fig. 5*B*).

We next examined the ability of the chimeric fusions to repress estradiol-dependent ER-transcriptional activity. Vectors expressing hER (pRST7-ER), HC-PR-A (pBKC-HC-PR-A), CH-PR-A (pBKC-CH-PR-A), hPR-A (pBKC-hPR-A), or cPR-A (pBKC-cPR-A), respectively, were cotransfected into HeLa cells (Fig. 5*C*) together with the ERE_3-TATA-LUC reporter. hER-mediated transcriptional activity was measured after 24 h in the presence of 10^{-7} M 17β-E_2 alone or in the presence of 10^{-7} M 17β-E_2 and 10^{-7} M R5020. As shown in Fig. 5*C*, HC-PR-A repressed hER-mediated transcriptional activity by 78%, and CH-PR-A repressed hER activity by only 30%. Once again wild type hPR-A was the strongest trans-repressor of hER activity, repressing hER activity by as much as 88%. Repression by cPR-A was only 18% in this experiment. Together these data suggest that the trans-repressor function of hPR-A is localized within the first 140 amino acids of the protein. Moreover, these data show that this 140-amino acid region is necessary for trans-repression of heterologous steroid receptor activity.

The N-terminal Repressor Region of hPR-A Is Not Sufficient for Trans-repression of hER Transcriptional Activity—To determine whether this 140-amino acid inhibitory region was necessary and sufficient for trans-dominant repression of heterologous steroid receptor activity, we transferred this region to the DNA binding domain of GAL4 (DBD-140NhPR-A) and assessed its ability to repress hER transcriptional activity (Fig. 6*B*). A similar GAL4-DBD fusion construct containing the N-terminal 90 amino acids of cPR-A was also made and used as a control. Western immunoblot analysis using the polyclonal hPR-A-specific antibody (B13-TK) and the monoclonal cPR-A-specific antibody (PR22) confirmed that these proteins were expressed intact and at similar levels to their cognate wild type receptors (data not shown). Expression vectors pBKC-DBD-140NhPR-A, pBCK-DBD-90NcPR-A, or pBKC-DBD (empty control plasmid) (Fig. 6*A*) were cotransfected into HeLa cells with an ERE_3-TATA-LUC reporter and the pRST7-ER expression vector. The cells were incubated with estradiol alone or estradiol and R5020 as before. It was observed that the GAL4-DBD fusions of the N terminus of the chicken and the human A isoforms of PR were unable to trans-repress hER activity under these conditions, whereas wild type hPR-A was capable of repressing hER activity by 80%. These observations suggest that the N-terminal repressor region of hPR-A acts only in the context of the full-length PR (either chicken or human) and that this region is necessary but not sufficient for trans-repression of hER transcriptional activity. More importantly, these results suggest that regions of the receptor other than the N terminus are required for trans-repression.

The B-upstream Sequence (BUS) of hPR-B Suppresses the Negative Effects of the N-terminal Repressor Region on AF-1 Function—One of the most interesting aspects of the experiments thus far is that we have mapped the inhibitory region of hPR-A to a domain that is wholly contained within hPR-A and hPR-B. Since the activation function in the N terminus, AF-1, is also contained within hPR-A, it suggests possibly that the "A" inhibitory region is dominant over the activation function contained within AF-1. Furthermore, it suggests that the role of BUS is to suppress the activity of the inhibitory region and permit AF-1 activity to be manifested. To test this hypothesis we created a series of fusion constructs by transferring various domains of hPR-B, hPR-A, and cPR-A to GAL4-DBD. These constructs are outlined in Fig. 7*A*. Western immunoblot analysis using B13-TK and PR22 confirmed the relative expression levels of these constructs (data not shown). Expression vectors pBK-DBD-140NhPR-A, pBK-DBD-90NcPR-A, pBK-DBD-NhPR-B, pBK-DBD-NhPR-A, pBK-DBD-AF-1, pBK-DBD-HBD, and pBK-DBD (containing the 140-amino acid repressor region of hPR-A, the N-terminal 90-amino acids of cPR-A, the N-terminal region up to its DBD of hPR-B, the N-terminal region up to its DBD of hPR-A, the 90-amino acid AF-1 region present in both isoforms of human PR, the C-terminal hormone binding domain (HBD) region also present in both receptors, and the GAL4-DBD domain, respectively) were cotransfected in HeLa and HepG2 cells. To access the transcriptional activity of these GAL4-DBD fusion constructs, we cotransfected them into mammalian cells with a $GAL4_5$-TATA-LUC reporter. After transfection the cells were induced for 24 h with two hormonal stimuli, no hormone and 10^{-7} M R5020, and then assayed for luciferase activity.

The results shown in Fig. 7*B* indicate that the 140-amino acid repressor region of hPR-A, the entire N-terminal region of hPR-A, and the 90-amino acid region of cPR-A have no activity on a GAL4-responsive promoter in either HeLa or HepG2 cells, whereas the whole N terminus of hPR-B, as well as the AF-1 and the HBD domains, displayed a significant increase in transcriptional activity (Fig. 7, *B* and *C*). AF-1 is more transcriptionally active in HepG2 (Fig. 7*C*), an AF-1-dominant cell line, than in HeLa cells (45- *versus* 5-fold). In contrast, the HBD of PR has greater activity in HeLa cells (Fig. 7*B*), an AF-2 dominant cell line (8- *versus* 1.8-fold) (30). From these observations we concluded that when tethered to AF-1, the 140-amino acid region of hPR-A (see NhPR-A fusion) is capable of repressing AF-1 activity (Fig. 7, *B* and *C*). Interestingly, by tethering the BUS region unique to hPR-B onto A-N (see NhPR-B fusion), it is possible to rescue the repressive effect of the 140-amino acid repressor region on AF-1 thus resulting in >20–30-fold activation of the GAL4-responsive promoter, in both cell lines. The observation that the transcriptional activity of the entire N terminus of B is more active than AF-1 alone suggests that in addition to overcoming the "A" repressive domain, the BUS region contains sequences that contribute to AF-1 activity (29).

DISCUSSION

The ability of progesterone to oppose estrogen action *in vivo* has been extensively documented. Progesterone abrogates estrogen induction by down-regulating ER protein concentration, decreasing the circulating estrogen levels (reviewed in Ref. 32), and antagonizing ER action at the molecular level. The mechanism of progesterone action on ER was first described in the mammalian uterus. These studies showed that uterine ER levels, of estrogen-treated rats, were decreased upon progesterone administration (33). Furthermore, it was reported that endometrial ER levels, in women undergoing curettage during the follicular phase of the menstrual cycle, could be decreased by administering medroxyprogesterone (a synthetic progestin) (34). More recently, studies done in breast cancer cells (35, 36) described a progesterone-mediated decrease in ER protein concentration due to decreased cellular ER mRNA levels, a direct result of inhibition of transcription of the ER gene.

Interestingly, progesterone is also capable of antagonizing ER-mediated regulatory events, although the molecular mech-

Transfections were normalized for efficiency using an internal β-galactosidase control plasmid. The data are presented as % activation, where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the presence of control vector alone or in the presence of hPR-A, ΔhPR-A, cPR-A, and ΔcPR-A, respectively, but in the absence of R5020. This value is independently calculated for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation was <10%.

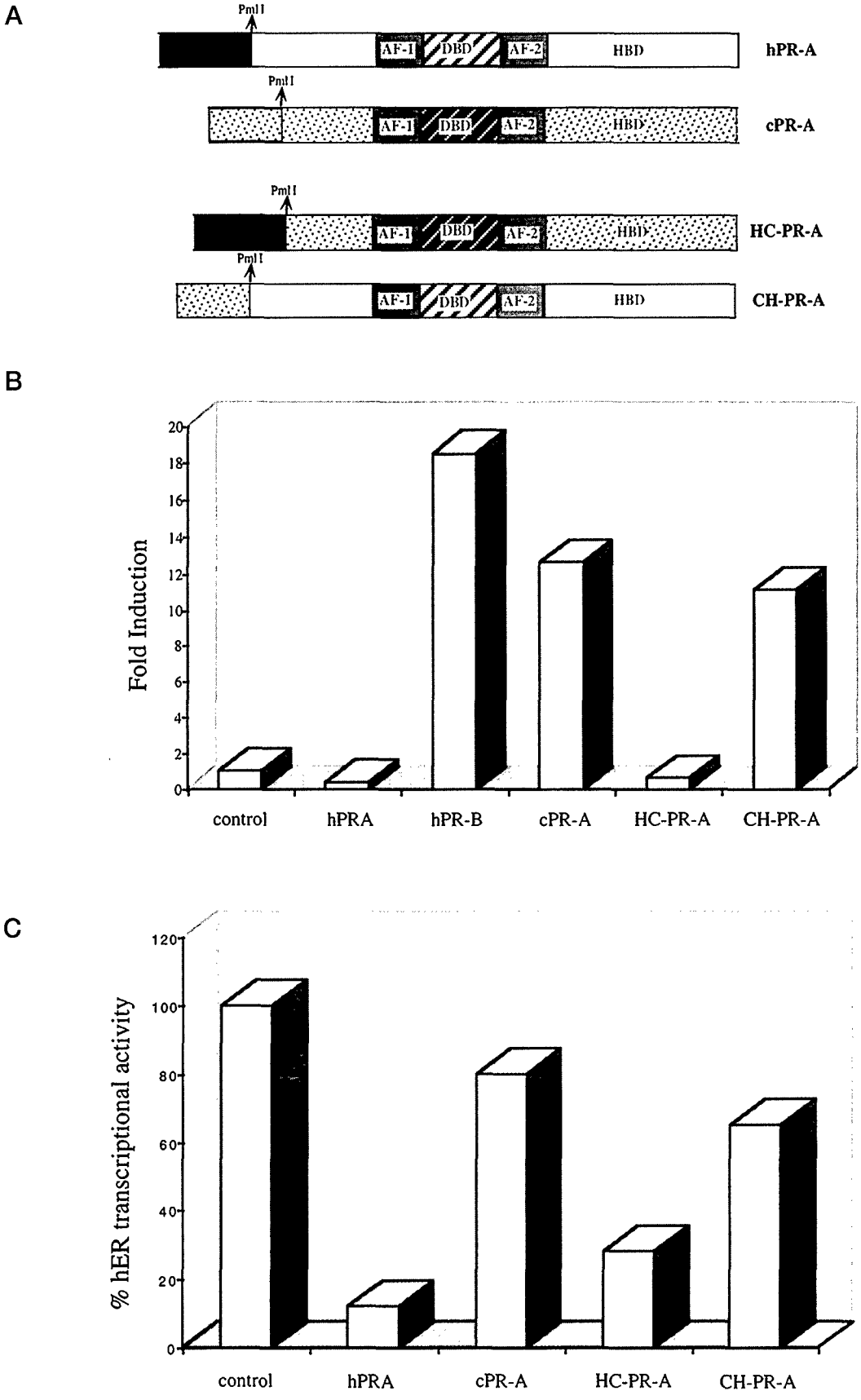

FIG. 5. **HC-PR-A is a potent repressor of hER transcriptional activity in HeLa cells.** *A*, the human/chicken chimeric constructs, HC-PR-A and CH-PR-A, were generated by swapping the N-terminal regions (upstream of the unique PmL1 restriction site, present in both receptors) of the human and the chicken receptors. *B*, HeLa cells were transiently transfected with vectors expressing hPR-A, cPR-A, HC-PR-A, and CH-PR-A, respectively. The transcriptional activity of these chimeric constructs was assayed on the PRE_3-TK, progesterone-responsive promoter. The activity was measured after 24 h induction with 10^{-7} M R5020. *Fold Induction* represents the normalized luciferase activity divided by basal (no hormone) activity, for each receptor-type after induction with ligand. *C*, HeLa cells were transiently transfected with vectors expressing the human estrogen receptor alone or in combination with a vector expressing hPR-A, cPR-A, HC-PR-A, and CH-PR-A, respectively. The transcriptional activity was

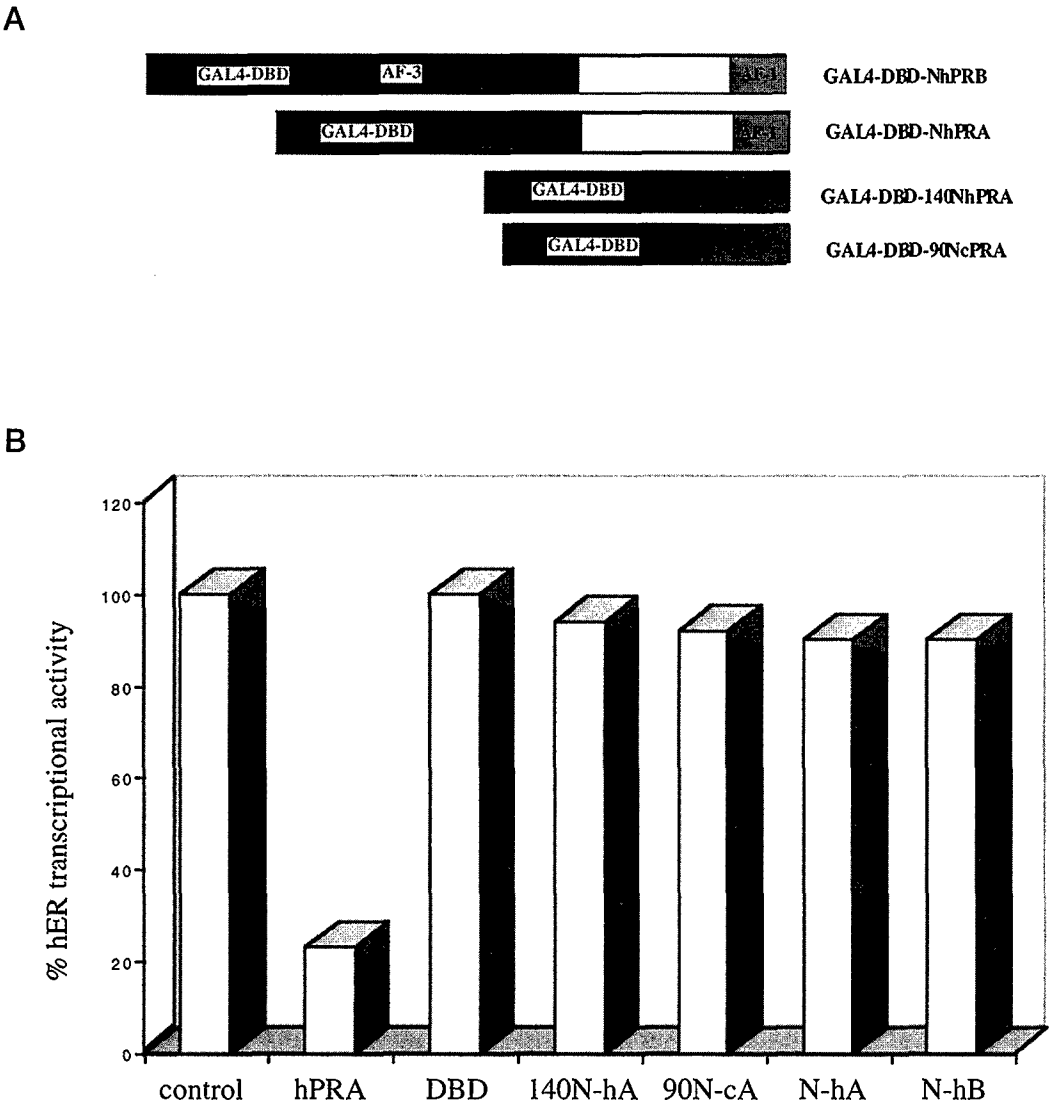

FIG. 6. **The N-terminal repressor domain of hPR-A is not capable of autonomous repression of hER transcriptional activity.** *A*, the GAL4 DNA-binding domain (GAL4-DBD) fusion constructs were made by transferring various N-terminal regions of hPR-B, hPR-A, and cPR-A onto GAL4-DBD. GAL4-DBD is depicted as a *solid black box* at the N terminus of these fusion constructs. *B*, HeLa cells were transiently transfected with vectors expressing the human estrogen receptor alone or in combination with a GAL4-DBD vector expressing GAL4-DBD fusions with various N-terminal regions of hPR-B (*DBD-NhPRB*), hPR-A (*DBD-NhPRA* and *DBD-140NhPRA*), or cPR-A (*DBD-90NcPRA*). The transcriptional activity was measured following the addition of 10^{-7} M 17β-estradiol and 10^{-7} M R5020 alone or in combination. A control was done in the absence of ligands. In these experiments estrogen receptor transcriptional activity was assayed on an ERE_3-TATA-LUC promoter. Transfections were normalized for efficiency using an internal β-galactosidase control plasmid. The data are presented as % activation where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the absence of R5020 for each data point. This value is calculated independently for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation was <13%.

anism of this antagonism is not completely understood. Various groups, including ours, have suggested that PR can antagonize ER transcriptional activity by sequestering a transcription factor necessary for proper ER action (18, 30, 37, 38). Specifically, we reported (18) that hPR-A but not hPR-B, in the presence of either progesterone or anti-progestins, inhibited ER-mediated transcriptional activity in transfected HeLa, CV-1, and HS578T cells but not in the HepG2 cell line. PR-A was also capable of antagonizing endogenous ER transcriptional activity when cotransfected with a simple estrogen-responsive promoter in MCF-7 breast cancer cells in the presence of RU486 (30). Others, however, have observed that hPR-B but not hPR-A was capable of repressing ER activity on a complex estrogen-responsive promoter (estrogen-responsive region on the pS2 gene) when transfected in MCF-7 cells (38). These noted discrepancies result most likely from differences in the

measured following the addition of 10^{-7} M 17β-estradiol and 10^{-7} M R5020 alone or in combination. A control was done in the absence of ligands. In these experiments estrogen receptor transcriptional activity was assayed on a ERE_3-TATA-LUC promoter. Transfections were normalized for efficiency using an internal β-galactosidase control plasmid. The data are presented as % activation where 100% represents a measure of 17β-estradiol-dependent transactivation by hER in the presence of hPR-A, cPR-A, HC-PR-A, and CH-PR-A, respectively, but in the absence of R5020. This value is independently calculated for each data point. Each data point represents the average of triplicate determinations of the transcriptional activity under given experimental conditions. The average coefficient of variation was <11% for both experiments.

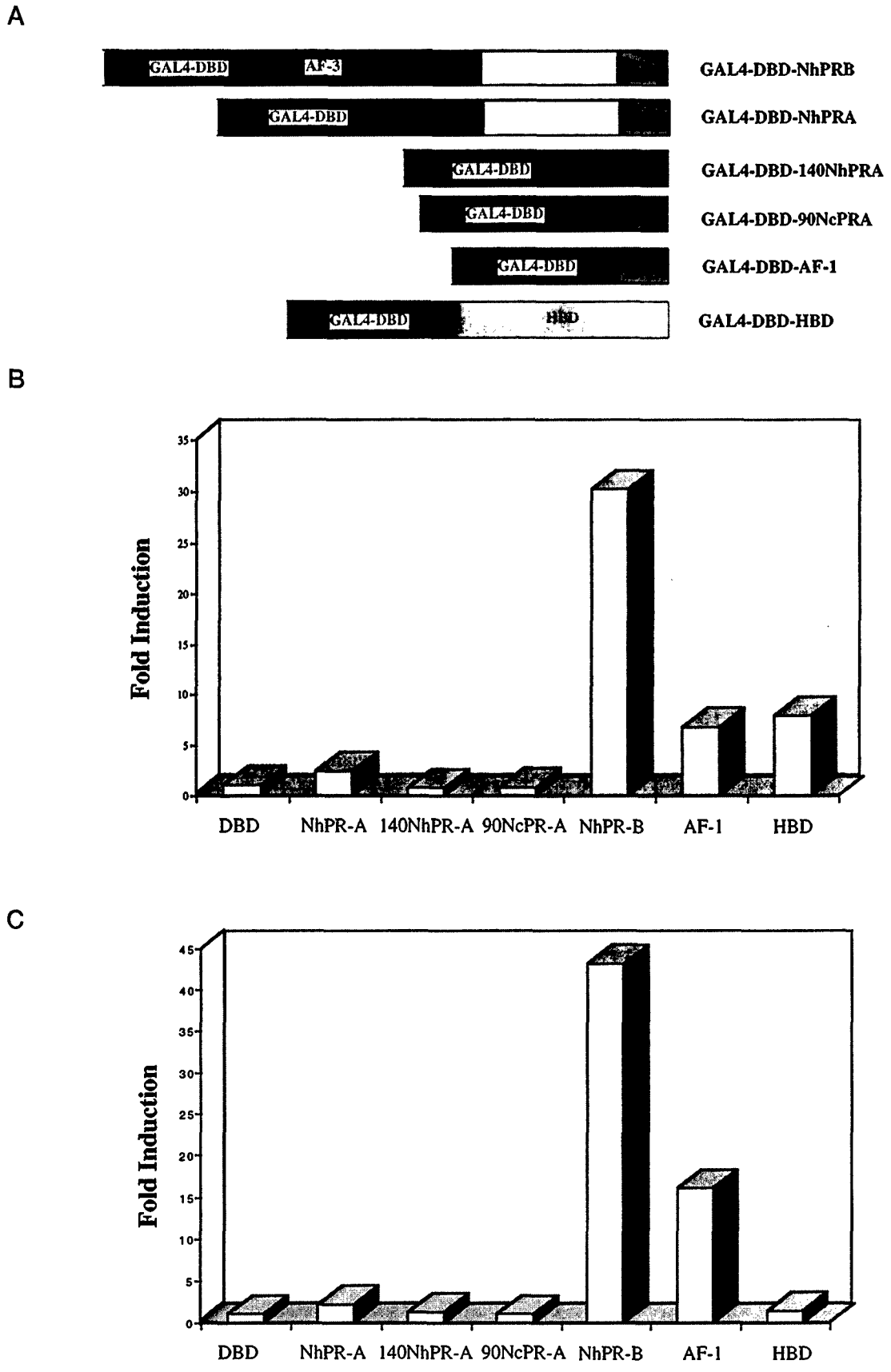

FIG. 7. **The N-terminal repressor region of hPR-A represses AF-1 activity and is itself antagonized by BUS.** *A*, the GAL4 DNA-binding domain (GAL4-DBD) fusion constructs were made by transferring various domains of hPR-B, hPR-A, and cPR-A onto GAL4-DBD. GAL4-DBD is depicted as a *solid black box* at the N terminus of these fusion constructs. HeLa cells (*B*) and HepG2 cells (*C*) were transiently transfected with expression vectors pBK-DBD-140NhPR-A, pBK-DBD-90NcPR-A, pBK-DBD-NhPR-B, pBK-DBD-NhPR-A, pBK-DBD-AF-1, pBK-DBD-HBD, or pBK-DBD together with a GAL4-responsive reporter plasmid, $GAL4_5$-TATA-LUC. The transcriptional activity was measured following the addition of 10^{-7} M R5020. The data are represented as *Fold Induction* in the presence of ligand *versus* absence of ligand for each triplicate data point. The average coefficient of variation was <12% for both experiments.

cell and promoter contexts used for analysis and in the relative expression of transcriptional co-factors and co-repressors.

Interestingly, hPR-A has also been reported to function as a strong trans-repressor of other steroid hormone receptor activity (17–19); however, the physiological importance of these observations remains to be determined. Furthermore, this dominant inhibitory action of hPR-A appears to be restricted to steroid hormone receptor-activated transcription as hPR-A is unable to antagonize vitamin D receptor activity and unable to modulate heterologous viral promoter activity (*i.e.* SV40, Rous sarcoma virus, and CMV) (17). Both PR and ER are involved in the maintenance and development of female reproductive tissues and more importantly are involved in the progression of hormone-dependent tumors of the breast (32). In addition, the co-expression of hER, hPR-A, and hPR-B in these tissues suggests that the mechanisms of action of these receptors might be linked. Thus there is a need to understand the precise molecular mechanism behind PR-mediated repression of ER transcriptional activity. We have previously proposed (30) that hPR-A may facilitate the cross-talk between progesterone and estrogen signaling pathways in progesterone and estrogen-responsive tissues. In support of our original hypothesis we showed that it is possible to antagonize endogenous ER transcriptional activity in MCF-7 cells by co-expression of hPR-A. In addition, the PR antagonist RU486 is capable of functioning as an antagonist of ER only in the presence of hPR-A. These actions of RU486 do not require the physical interaction with hER and are likely mediated by a non-competitive mechanism of action of RU486. It is possible then that the clinical importance of RU486 in the treatment of endometriosis, uterine fibroids, brain meningiomas, and hormone-dependent breast cancers may well be a result of its ability to function as an anti-progestin as well as an anti-estrogen.

This study defines the structural differences between hPR-A and hPR-B that confer to the A isoform the ability to transrepress hER transcriptional activity. Previously, it has been postulated that the differences in the transcriptional activities of the two isoforms of the human PRs were due to unique sequences present in hPR-B (29). However, the observation that cPR-A is also an activator of progesterone-responsive promoters but lacks the activating B-specific sequences suggested to us that something unique to hPR-A is responsible for the differences in the transcriptional activities of the two human receptors. Sequence analysis of the human and chicken A-receptors revealed that the proteins differed in their N termini. It follows that the structural difference between the human and the chicken A isoform of PR confers to the human A receptor the ability to trans-repress steroid hormone receptor transcriptional activity. Here we show that only hPR-A but not hPR-B or cPR-A is capable of opposing ER-mediated transcriptional activity and that the N-terminal 140-amino acid region of hPR-A is responsible for this repressor activity.

Furthermore, our observation that the repressor region of hPR-A is necessary but not sufficient for trans-repression of heterologous steroid receptor activity suggests that regions of the receptor other than the N terminus of hPR-A are required for trans-repression. In support of this hypothesis it has recently been shown that the N terminus of hPR-A and its C-terminal hinge region interact when assayed *in vitro* (39). Thus, it is possible that sequences within the N terminus and the C terminus of hPR-B together form a surface that allows the receptor to interact with required transcription co-factors. Given this information, and that presented in this paper, it would appear that the simplest model to explain the differential activity of hPR-A and hPR-B is that both receptors compete for a limiting pool of co-factors and that the complex formed with hPR-A is transcriptionally inactive but represses transcription by sequestering a transcription factor required by hPR-B. However, this simple model is unlikely to be completely correct. In previous work, we demonstrated that the ability of hPR-A to inhibit hER transcriptional activity in a hormone-dependent manner occurred independently of the relative expression of the two receptors and was dependent on the absolute level of hPR-A. This would seem to rule out a classical squelching model. It suggests instead that the inhibitory activity of hPR-A occurs through a totally independent pathway. Our working model at the current time is that the interaction between sequences within the hPR-B BUS region permit PR-AF-1 to interact with cellular transcription factors within the cell which are different from those that interact with hPR-A. Specifically, we propose that in the presence of hormone hPR-B can interact with the co-factors required for transcriptional activity. On the other hand hPR-A may interact with a different subset of proteins and form a complex that can interfere with ligand-dependent transcriptional activity of all the steroid receptors. Although this model can only be tested upon the isolation of the PR-A- and hPR-B-associated proteins, the observation that the inhibitory activity of hPR-A occurs in a cell-restricted manner supports this model.

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